THE EFFECTS OF AGING ON ELECTROPHILE AND EPOXIDE METABOLISM BY HEPATIC AND PULMONARY CYTOSOLIC GLUTATHIONE S-TRANSFERASES

By

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This dissertation is dedicated to my parents, Neil and Mary, for having me, keeping me, supporting me, and letting me leave. In addition, it is dedicated to Herman and Priscilla; may He grant them peace and love to reward their sacrifices on my behalf. This is also dedicated to Ferdinand Feghoot, and that facet of the man which sustained me throughout. And finally, it is dedicated to the loving memory of Friederich Nietzsche who taught us that "that which does not kill us makes us stronger."

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PREFACE

Age will not be defied.

Francis Bacon, Essay 30, Regimen of Health

This dissertation is composed of an introduction, three chapters written in a standard manuscript style, and a general conclusions section. Unfortunately, some material is redundant, but is germane to more than one section. It is hoped that the reader will accept the repeated material as being important in the scope of this dissertation.

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THE EFFECTS OF AGING ON ELECTROPHILE AND EPOXIDE METABOLISM BY HEPATIC AND PULMONARY CYTOSOLIC GLUTATHIONE S-TRANSFERASES

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The cytosolic glutathione S-transferases are a family of enzymes that conjugate the cellular nucleophile glutathione with electrophiles, such as epoxides. Changes in these enzymes in the liver or lung may thus alter the susceptibility to toxic effects of these electrophiles.

With liver cytosol from male Fischer 344 rats, specific activities with all epoxide substrates tested were lower in the senescent group than in the young one. With lung cytosol from males and liver and lung cytosol from females, specific activities declined with only some of the substrates. The increasing apparent K_M, measured with styrene oxide, of liver and lung cytosol from senescent males indicated that these tissues had decreased abilities to metabolize the low concentrations of epoxides that might be found with environmental exposure. Finally, age-related changes in organ weights and protein contents altered both the total metabolic rates and those per gram of tissue. Thus, tissue-, sex-, and substrate-specific alterations in metabolism occurred during aging.

In addition, altered metabolism of other electrophilic substrates indicated that isozyme-specific changes occurred during aging. Column isoelectric focusing and cytosol immunotitration experiments revealed that changes in isozyme concentrations occurred with increasing age; the above changes were not due to alterations in the specific activities of purified isozymes. In liver cytosol, concentrations of isozymes E, C, and B increased by middle-age and decreased by senescence (relative to the young group), whereas they followed a pattern of steady decrease in lung cytosol. With liver cytosol, concentrations of isozymes A and AA changed only slightly by middle-age and then increased by senescence. In lung cytosol, which contained no isozyme AA, concentrations of isozyme A did not change. These data indicated that in liver cytosol, the Y_a and Y_c and the Y_b and Y'_b subunits increased similarly in middle-age and decreased in senescence (relative to the young group). In lung cytosol, all followed patterns of steady decrease. Thus, tissue-specific, and presumably sex-specific, alterations occurred during the aging process in the concentrations of glutathione S-transferase isozymes. Aging may therefore alter metabolism of toxic electrophiles and epoxides.

INTRODUCTION

With the exception of some parenterally administered drugs, most xenobiotics of pharmacological or toxicological interest are relatively hydrophobic compounds which are readily able to diffuse passively across membranes and lipid barriers. The hydrophobic xenobiotics must be metabolized to more hydrophilic compounds to facilitate excretion. The nature of these metabolic, or biotransformation, events has stimulated many investigations. From studies dealing with events at the molecular level (such as the forces of xenobiotic binding to the active site of an enzyme or the electron transport to this site) to studies of gross toxicological effects of exposure to the xenobiotic, biomedical science has focused on the nature of the events and the consequences of xenobiotic biotransformation.

In more recent years, intense interest has developed in studying the capacities of biotransformation during the stages of early development and during the process of senescence. Some researchers have recognized that during the differential development of enzyme systems it is possible to differentiate between fast- and slow-developing enzyme systems. As a result, it is possible to study *in vivo* a system analogous to a semipurified enzyme system. Similarly, differential alterations in enzymes during the process of aging (in this dissertation aging will refer to the process of senescent aging) may afford an opportunity to study the effects of enzyme activity less affected by senescence in comparison to activity from one which is more affected. In addition, the toxicological significance of the simultaneous existence of immature or partially inactive enzymes along with the more active ones may be of interest. It was with these considerations that this project was initiated: the project to study senescent alterations in the glutathione S-transferases in liver and lung with subsequent alterations in epoxide metabolism.

Many biotransformation enzymes have been studied in aging mammals, especially those of microsomal origin. Several groups have examined NADPH:cytochrome c reductase or NADPH:cytochrome P-450 reductase. Schmucker and Wang (1) used liver microsomes from 1-, 16-, and 27-month-old Fischer 344 (F344) rats and found that NADPH:cytochrome c reductase activity was 73 ± 8, 102 ± 10, and 45 ± 20 nmol/min/mg of microsomal protein, respectively. Birnbaum and Baird (2) reported that in liver microsomes from 3- and 28-30-month-old male CFN rats that NADPH:cytochrome c reductase activity was 58.6 ± 2.3 and 58.4 ± 1.8 nmol/min/mg microsomal of protein, respectively. Thus, they found no change with aging. However, they reported (3) that in liver microsomes from male CFN rats of 3-, 12-, and 27-months-old that NADPH:cytochrome P-450 reductase activities were 96 ± 9, 66 ± 6, and 60 ± 6 units (Δ_{OD} 450 nm)/min/mg of microsomal protein, respectively. Kao and Hudson (4) reported that with liver microsomes from male F344 rats of 10 or 100 weeks of age values of NADPH:cytochrome c reductase were 265.7 ± 19.6 and 178.3 ± 14.0 nmol/min/mg of microsomal protein. Finally, Rikans and Notley (5) reported that in liver microsomes from male F344 rats of 3-5, 14, or 24 months of age, NADPH:cytochrome _c_ reductase values were 240 ± 10, 140 ± 10, and 120 ± 10 nmol/min/mg of microsomal protein, respectively. Thus, the general consensus is that NADPH:cytochrome _c_ or cytochrome P-450 reductase values tend to be highest in young (1-5-month-old) animals and decline significantly by old age (24-30-months-old).

The activity of heme oxygenase, an enzyme involved in heme breakdown, has also been shown to decline with increasing age. Ariyoshi _et al_. (6) report that in liver microsomes from male Wistar rats of 30, 100, or 300 days of age, activities were 1.41 ± 0.24, 0.82 ± 0.08, and 0.45 ± 0.04 nmol of bilirubin produced per min per mg of microsomal protein, respectively.

The activities of a number of other microsomal drug-metabolizing enzymes have also been studied. Kao and Hudson (4) reported that in liver microsomes from male F344 rats of 10 and 100 weeks of age, benzphetamine N-demethylase activities were 4.48 ± 0.32 and 1.45 ± 0.20 nmol/min/mg of microsomal protein, respectively. Similarly, Birnbaum and Baird (2) reported that in liver microsomes from male CFN rats of 3 and 28-30 months of age, benzphetamine N-demethylase activities were 5.64 ± 0.31 and 2.94 ± 0.49 nmol/min/mg of protein. Rikans and Notley (5) reported that in liver microsomes from male F344 rats of 3-5, 14, and 24 months of age, benzphetamine N-demethylase activities were 6.08 ± 0.10, 10.06 ± 0.33, and 2.26 ± 0.16 nmol/min/mg of protein, respectively.

Birnbaum and Baird (3) reported that in liver microsomes from male CFN rats, benzo[a]pyrene hydroxylase activities from 3-, 12-, and 27-month-old animals were 94.2 ± 8.2, 112.2 ± 12.2, and 55.4 ± 7.6 units (relative fluorescence)/10 min/mg of microsomal protein. They also reported (2) that ethylmorphine N-demethylase activity in liver microsomes from 3- and 28-30-month-old male CFN rats decreased from 3.90 ± 0.45 to 1.29 ± 0.11 nmol/min/mg of microsomal protein, respectively.

Kao and Hudson (4) reported that in liver microsomes isolated from male F344 rats of 10 and 100 weeks of age, a significant change in 7-ethoxycoumarin O-deethylase activity from 2.42 ± 0.32 to 1.87 ± 0.21 nmol/min/mg microsomal protein occurred.

Rikans and Notely (5) reported that in liver microsomes from male F344 rats of 3-5, 14, and 24 months of age, aniline hydroxylase activities were 0.90 ± 0.03, 0.89 ± 0.03, and 0.67 ± 0.04 nmol/min/mg of microsomal protein, respectively.

Birnbaum and Baird (7) reported that in liver microsomes from male CFN rats of 3, 12, and 25-27 months of age, epoxide hydrolase activities were 9.83 ± 0.82, 13.21 ± 1.09, and 21.70 ± 1.53 nmol of styrene glycol formed per min per mg of microsomal protein, respectively. In liver microsomes from C57BL/6J mice (same age groupings), activities were 8.46 ± 0.51, 6.46 ± 0.25, and 11.84 ± 0.71, respectively. Thus, epoxide hydrolase did not seem to follow the pattern of many other microsomal enzymes of having the highest activity in young animals and the lowest in senescent animals.

Baird et al. (8) showed that senescence in male CFN rats affected hexobarbital sleeping times. If a dose of 100 mg/kg was given intraperitoneally to 140-, 570-, and 940-day-old animals, the sleeping times were 22, 29, and 92 minutes, respectively. Thus, the in vitro effects on drug metabolism seen with other substrates may have an in vivo effect, as seen here.

Knook (9) showed that nonmicrosomal enzymes in hepatocytes are affected by age. Acid phosphatase activities in hepatocytes isolated from female BN/Bi rats of 3, 12, 24, and 30-35 months of age were 63.5 ± 4.9, 67.7 ± 15.8, 118.8 ± 9.7 and 78.1 ± 8.6 nmol of 4-methylumbelliferone per min per 10^6 cells, respectively. In the same age groupings, arylsulfatase B activities were 13.4 ± 0.5, 21.6 ± 4.8, 33.5 ± 1.1, and 16.9 ± 3.4 nmol of nitrocatechol per min per 10^6 cells, respectively.

As with the age-related alterations in microsomal metabolism, those in cytosolic glutathione S-transferase activities appear to be substrate-specific. Stohs et al. (10) reported that in liver cytosol from female Swiss-Webster mice of 1, 3, 9, 15, and 18 months of age, activities toward 1,2-dichloro-4-nitrobenzene (DCNB) were approximately 45, 105, 145, 40, and 40 nmol of conjugate formed per min per mg of protein, respectively. However, Birnbaum and Baird (7) reported that there were no significant age-related changes in glutathione S-transferase activity toward styrene oxide (STOX) when liver cytosol from male CFN rats of 3, 12, and 27 months of age was used. They reported values of 13.14 ± 0.57, 11.49 ± 1.17, and 13.25 ± 0.45 nmol/min/mg of protein, respectively. In C57BL/6J mice of the same age groupings, values were 11.45 ± 0.67, 12.19 ± 1.01, and 9.80 ± 1.23 nmol/min/mg of protein, respectively.

Similarly, Wilson et al. (11) reported that in hepatocytes isolated from female BN/Bi rats of 3 or 24 months of age, activities toward 1-chloro-2,4-dinitrobenzene (CDNB) were 333 ± 92 and 344 ± 111 µmol/hr/mg of protein. In hepatic sinus lining cells from these same animals, activities were 102 ± 21 and 90 ± 23 µmol/hr/mg of protein. Thus, age-related alterations in glutathione S-transferase activity seem to be substrate specific.

A recent study by Chiang et al. (12) reported age-related alterations in two isozymes of cytochrome P-450 from livers of male New Zealand White rabbits of various ages. While total cytochrome P-450 declined from a high value of about 1.3 nmol/mg of microsomal protein at 50 days of age to about 1.0 nmol/mg microsomal protein at 800 days of age, cytochrome P-450 LM_4 (the major 3-methylcholanthrene-inducible form) was only slightly decreased. However, cytochrome P-450 LM_2 (the major phenobarbital-inducible form) levels decreased steadily after a peak at 50-100 days of age. Thus, isozymes of cytochrome P-450 appear to be differentially affected by aging.

Whether various isozymes of the glutathione S-transferases are similarly differentially affected by aging is at present unknown. However, the age-related alterations in DCNB metabolism without concomitant changes in CDNB or STOX metabolism would imply that selective age-related isozyme-specific changes do indeed occur. Results presented in this dissertation define the age-related changes in these isozymes.

Various studies of age-related alterations in drug metabolism in humans have been published. Clearance of antipyrine from the plasma was studied by Wood _et al_. (13) and Greenblatt _et al_. (14). Wood _et al_. (13) reported that in adult male smokers antipyrine plasma clearance fell from a mean of 0.59 ± 0.06 ml/kg/min in the group below 40 years old to less than 0.40 ml/kg/min in the group above 40 years old. However, in nonsmokers, no such age-related change was evident. They concluded that the ability of smoking to induce hepatic drug metabolizing enzymes decreased with increased age. However, Greenblatt _et al_. (14) reported that in males of age 23-43, plasma clearance of antipyrine was 0.81 ml/kg/min whereas in males of age 60-76, clearance was 0.49 ml/kg/min. Similarly, in females, clearance was 0.64 ml/kg/min in the 22 to 33-year-old age group and 0.49 ml/kg/min in the 62 to 84-year-old group. Thus, a significant decrease in antipyrine clearance with aging occurred in both males and females; however, smokers and nonsmokers were not considered separately.

Vestal _et al_. (15) studied propranolol disposition in adult males of 21-73 years of age. By giving a small amount of radiolabeled propranolol in the seventh dose, they found that the plasma half-time of propranolol was 4.19 ± 1.38 hr in the group under 35 years of age whereas it was 5.05 ± 1.36 hr in the group above 35 years of age. If nonsmokers and smokers were considered separately, it was found that nonsmokers had 200% higher blood levels of propranolol than smokers, but no change in plasma half-life was evident. Also, the older age group had significantly higher plasma propranolol levels than either young nonsmokers or smokers. These data indicate that propranolol-metabolizing enzyme activities are decreased with increasing age.

Theophylline clearance is also significantly affected by aging (16). As with antipyrine and propranolol, theophylline can be metabolized by oxidative reactions. In age groups of below 20, 20-39.9, 40-59.9, and above 60 years old, clearance was 91.9 ± 28.1, 64.2 ± 27.2, 47.8 ± 24.8, and 43.1 ± 23.2 ml/kg/hr, respectively.

Lorazepam is a benzodiazepine which is eliminated as the glucuronide conjugate. Greenblatt et al. (17) compared two groups of males aged 19-38 and 60-84 years and found no significant change in volume of distribution (1.11 and 0.99 L/kg) or elimination half-time (14.1 and 15.9 hr). However, total clearance was significantly decreased in the aged group from 0.99 to 0.77 ml/kg/min.

Another benzodiazepine, nitrazepam, is metabolized by nitroreduction. Kangas et al. (18) studied the elimination kinetics in males aged 13-38 and 66-94 years. After a single oral dose, the beta phase of elimination was examined. The volumes of distribution were 2.4 ± 0.8 and 4.8 ± 1.7 L/kg; the half-times were 28.9 ± 7.4 and 40.4 ± 16.2 hr; and the beta elimination rate constants were -0.0257 ± 0.0074 and -0.0198 ± 0.0080 hr^{-1}, respectively.

Farah et al. (19) could find no age-related alterations in isoniazid metabolism. Isoniazid is metabolized by acetylation. In age groups of 20-35 and above 65 years, fast acetylators had isoniazid plasma half-times of 1.4 and 1.5 hr, respectively. Slow acetylators had plasma half-times of 3.7 and 4.2 hr, respectively.

In both laboratory animal and human studies, alterations in drug metabolism or disposition during the process of aging appear to be specific for the type of xenobiotic under study. Perhaps as suggested by Chiang *et al*. (12) alterations, or lack thereof, in levels of isozymes of multiple enzyme systems occur.

What are the consequences of age-related alterations in xenobiotic metabolism? Depending upon the properties of the xenobiotic, consequences can be beneficial, insignificant, or toxic. For drugs such as isoniazid, with no demonstrable alteration in disposition with aging, little additional significant risk from the drug should be expected in the elderly population (provided that therapeutic measures such as pyridoxine administration are followed). For potentially carcinogenic xenobiotics which require bioactivation to an ultimate carcinogenic form, as benzo[*a*]pyrene (by microsomal hydroxylases and epoxide hydrolase) (20) or dibromoethane (by glutathione S-transferases) (21), age-related declines in metabolism could have significant effects.

Baird and Birnbaum (22) studied production of mutagenic metabolites from benzo[*a*]pyrene and 2-fluorenamine with the 9000x*g* supernatant fraction from livers of 300- and 700-day-old male CFN rats. In the Ames *Salmonella typhimurium* mutagenesis test they found these results:

Strain	Age in days of CFN rat	Revertants/Plate Control	Benzo[*a*]pyrene	2-Fluorenamine
TA 98	300	19	262	285
TA 98	700	20	508	995
TA 100	300	45	245	439
TA 100	700	50	302	830

Clearly the 9000x*g* supernatant fractions from the older rats generated a greater amount of mutagenic metabolites from both benzo [*a*]pyrene and 2-fluorenamine than did those from the younger rats. The *S. typhimurium* TA 98 strain is sensitive to frame-shift mutations whereas the TA 100 strain is sensitive to base-substitution mutations.

Jayaraj and Richardson (23) reported that the production of mutagenic metabolites from aflatoxin B_1 decreased with increasing age. Liver microsomes from male F344 rats of 12, 18, and 27 months of age were used in the Ames test with *S. typhimurium* TA 98. Incubations with microsomes from these age groups produced 2196 ± 358, 1025 ± 245, and 902 ± 243 revertants per plate per mg microsomal protein, respectively. These results expressed per nmol cytochrome P-450 were 9404 ± 1103, 8540 ± 1020, and 7633 ± 868 revertants per plate per nmol cytochrome P-450, respectively. The trend of a decreasing production of mutagenic metabolites from aflatoxin B_1 with increasing age is more evident when the data are expressed per mg microsomal protein.

Birnbaum and Baird (7) studied the binding of benzo[*a*]pyrene to DNA in presence of liver microsomes from 12- and 25-27-month-old male C57BL/6J mice and CFN rats. In mice, 1.3 and 2.0 nmol of benzo[*a*]pyrene were bound per mg of calf thymus DNA per mg of microsomal protein when microsomes from the young and old groups were used, respectively. In the rats, DNA binding was reported to be 50% greater with microsomes from the old rats.

One reasonable explanation for the age-related differences in mutagenic potency of benzo[*a*]pyrene could be alterations in the cytosolic glutathione S-transferases. It has been reported that glutathione S-transferases A, B, C, or E can increase the levels of benzo[a]pyrene-glutathione conjugates and decrease DNA binding (24,25).

Clearly the production of mutagenic metabolites is substrate dependent and age-related alterations may occur. In addition, the possible toxicological consequences of this altered production of mutagenic metabolites should include consideration of age-related alterations in DNA repair processes. As reported by Baird and Birnbaum (22) and Birnbaum and Baird (7), there was an increased production of mutagenic benzo[a]pyrene metabolites and an increased level of DNA binding of these metabolites with the process of aging. Thus, a critical question is whether the ability to repair DNA lesions is altered in senescence.

Before reviewing the data regarding this question, it may be helpful to discuss the types of DNA repair processes. First, thymine dimer damage may be repaired by a photoreactivation enzyme. This enzyme uses near-visible ultraviolet (UV) light to convert the dimers to monomers. This mechanism is nearly error-free since there is no breakage of the DNA backbone at any time. Nucleotide excision is perhaps the most common mechanism which is used to repair chemically-induced damage. In this process, a nick or incision is made near the site of the damage; an exonuclease cleaves the damaged base plus a length of undamaged bases. A DNA polymerase then replaces the cleaved bases by matching them with bases on the complementary strand and a DNA ligase closes the nick. While this is considered a damage repair process, it must be realized that it involves a break in the DNA-phosphate backbone and that some errors may occur. Base excision is a process in which only the damaged base, and not the sugar-phosphate, is removed by a glycosylase, after which an insertase attaches the appropriate free-base. This process is believed to function in the repair of methylated bases (as the N^3 of adenine or O^6 of guanine). Little is currently known about the error rate of this process. Both single- and double-stranded breaks may occur after exposure to radiation or damaging chemicals. DNA ligase can close some of the single-strand breaks. Presently it is not known how double-strand breaks are repaired (26).

The important considerations in DNA repair are the type of damage (such as methylation, dimerization, or nicks), the type of repair process and its error rate, and the replicative frequency of the tissue. Damaging events in cells that are rapidly dividing (such as epithelial and stem cells) are more likely to be "set" than those in slowly dividing cells (such as neural cells). This is because of the possibility of a replication fork reaching a damaged base before a repair enzyme does, with the resulting inappropriate pairing of a new base.

Niedermuller (27) administered N-methyl-N-nitrosourea (MNU), methyl methanesulfonate (MMS), and N,N-dimethylnitrosamine (DMN) intraperitoneally to male Sprague-Dawley rats 6 and 24-26 months of age. Repair of DNA damage was monitored as the incorporation of $[^3H]$thymidine deoxyribonucleotide in unscheduled DNA synthesis (UDS, or repair synthesis). Although some tissues showed variable results, the 6-month-old group had higher rates of UDS than the 24-26-month-old group with MNU, MMS, and DMN. In no case did the old group have a higher rate of UDS.

Human cells also show age-related declines in UDS. Bowman et al. (28) and Icard et al. (29) monitored UDS in WI38 cells (nontransformed human lung fibroblasts). With increasing passage number, the rate of repair of UV light-induced damage, as measured by UDS, was significantly reduced.

Peripheral leukocytes from individuals 13-94 years of age were studied by Lambert et al. (30). After irradiation with UV light, UDS was measured as incorporation of deoxythymidilic acid. In leukocytes from individuals from age 23 to 82, incorporation decreased from 897 cpm/10^6 cells to 450 cpm/10^6 cells, a highly significant change.

Thus, in both rats and humans, an age-related decline in DNA repair synthesis occurs. The age-related alterations in drug metabolism and in the production of mutagenic metabolites assume a greater level of importance with this lower level of repair. In addition, the glutathione S-transferases have been shown to play a role in the detoxification of reactive xenobiotics and may moderate the quantity of DNA binding by these chemicals.

The glutathione S-transferases are a group of predominantly cytosolic (although some microsomal glutathione S-transferases are now known to exist) enzymes which function in a number of biological reactions. Many of these reactions require glutathione (GSH). In the liver of rats, at least seven isozymes of the glutathione S-transferases exist, glutathione S-transferases AA, A, B, C, D, E, and M (31). In the human liver, glutathione S-transferases alpha, beta, gamma, delta, epsilon, omega, psi, and mu have been purified (32-34). In the rat, only isozymes A and C are immunologically cross-reactive.

In the human, isozymes alpha, beta, gamma, delta, and epsilon are immunologically cross-reactive, and omega and psi cross-react with antibodies prepared to the other. In addition, glutathione S-transferase rho has been purified from human erythrocytes and glutathione S-transferase pi has been found in the human placenta (35,36).

The reactions catalyzed by the glutathione S-transferases are many and isozyme-specific. Selenium-independent glutathione peroxidase activity, as quantitated with organic hydroperoxides such as cumene hydroperoxide, is highest with glutathione S-transferases AA and B; A and C have low but measurable activity; E has still lower activity (37,38).

Glutathione S-transferase B has the highest activity of the rat liver isozymes as the Δ^5-3-ketosteroid isomerase. In this reaction, the 5-6 double bond of Δ^5-androstene-3,17-dione is converted to a 4-5 double bond. GSH is required and believed to be reversibly conjugated, although it is not consumed by the reaction (39,40). In the human, glutathione S-transferases gamma, delta, and epsilon are more active than alpha and beta as the isomerase.

The ability of the glutathione S-transferases to noncovalently bind bilirubin, heme, certain steroids, some carcinogenic azo-dyes (such as 4-dimethylaminoazobenzene), bromosulfophthalein, and indocyanine green was variably attributed to ligandin, Y protein, azo-dye carcinogen binding protein, and other intracellular proteins. In fact, glutathione S-transferase B has been referred to as the "intracellular albumin" of the liver (41). Habig *et al*. (42) first identified ligandin as being glutathione S-transferase B.

In addition to the peroxidase, isomerase, and binding properties discussed above, the glutathione S-transferases have activity as conjugating enzymes. Substrate specificity frequently overlaps for both the rat and human isozymes. In the human, the following activities with DCNB, CDNB, *p*-nitrobenzyl chloride (PNBC), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP), and benzo[*a*]pyrene (BZAP), are (rates in µmol/min/mg of protein)

Isozyme	DCNB	CDNB	PNBC	EPNP	BZAP
alpha	0.049	19	0.17	0	<0.030
beta	0.065	16	0.22	0	<0.030
gamma	0.035	17	0.019	0	<0.030
delta	0.050	37	0.20	0	<0.030
epsilon	0.043	34	0.16	0	<0.030
omega	n.r.	n.r.	n.r.	n.r.	n.r.
psi	n.r.	n.r.	n.r.	n.r.	n.r.
mu	0.032	187	n.r.	0.11	0.92
rho	0.025	66	n.r.	0	0
pi	0.11	105	n.r.	0.37	0.13

(n.r. indicates values not reported)

In the rat, activities with these substrates, plus those with *trans*-4-phenyl-3-buten-2-one (TPBO), are

Isozyme	DCNB	CDNB	PNBC	EPNP	BZAP	TPBO
AA	0.008	14	0.09	n.r.	0.004	n.r.
A	4.3	62	11.4	0.1	0.087	0.02
B	0.003	11	0.1	0	0.011	0.001
C	2.0	10	10.2	0	0.098	0.40
D	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
E	<0.0001	0.01	4.1	6.7	0.069	0
M	0.004	n.r.	0.5	0	n.r	n.r.

Clearly, some overlap in substrate specificity exists with both the rat and human glutathione S-transferase isozymes. This overlap in specificity may be in part accounted for by the two subunits which compose the isozymes. All of the rat and human isozymes are composed of two subunits, each of 23,000-25,000 daltons. In the rat, isozyme B is believed to be composed of a small (23,000 dalton) and large (25,000 dalton) subunit. Many groups, such as Pickett et al. (43,44), Beale et al. (45) and Mannervik and Jensson (46) define these as Y_a (small subunit) and Y_c (large subunit). Unfortunately, Bhargava et al. (47) defined these two subunits as Y_a and Y_b. However, the designation as Y_a and Y_c seems to be more accepted. Several groups have also proposed that glutathione S-transferase B may also be a homodimer composed of Y_aY_a (48,49). According to Mannervik and Jensson (46) the glutathione S-transferases are composed as follows: AA, Y_cY_c; A, Y_bY_b; B, Y_aY_c; and C, $Y_bY'_b$. The Y_b subunit has a molecular weight of 23,500 daltons.

Dierickx and Yde (50) showed that sulfanilic, metanilic, and orthanilic acids and their N-acetylated forms can inhibit glutathione S-transferase activity of isozymes AA, A, B, C, E, and M toward CDNB. The binding of these aminobenzenesulfonic acids and their derivatives inhibited activity to various degrees. In addition, with glutathione S-transferase A, measurement of the K_m for GSH (by use of a constant concentration of DCNB) gave a biphasic Lineweaver-Burk plot (51). These facts taken together indicate that not only do multiple glutathione S-transferase catalytic and binding sites exist, but they likely have different affinities (K_m) and maximal rates (V_{max}), depending upon the substrates being tested. Bilirubin binds to a high-affinity site ($K_a = 5 \times 10^7$ M^{-1}) and a low affinity site

(K_a = 3 x 10^5 M^{-1}) (52). Binding to the high affinity site does not affect glutathione S-transferase activity. In fact, only the smaller, or Y_a subunit, of glutathione S-transferase B binds bilirubin (46), whereas both subunits have glutathione S-transferase activity toward CDNB. Activity of glutathione S-transferase B toward CDNB was reduced from 11 to 4 µmol/min/mg of protein with a saturating concentration of bromosulfophthalein, implying that this remaining activity was associated with the larger subunit (47). Homogeneous preparations of the smaller Y_aY_a subunits had high glutathione S-transferase activity. Thus, both subunits apparently have catalytic and binding sites differing in activity and affinity.

The above results show that some confusion exists as to the precise subunit composition of the isozymes. For the sake of clarity, the nomenclature scheme proposed first by Jakoby, namely AA, A, B, C, D, E, and M, will be followed.

This nomenclature has been adopted in other rat tissues, such as the lung. By the use of affinity chromatography followed by hydroxylapatite gel chromatography, five glutathione S-transferase isozymes were purified from the lungs of male Sprague-Dawley rats. Three of these isozymes were identified as glutathione S-transferases A, B, and C by substrate specificity and immunological cross-reactivity. Another form appeared to have resulted from blood which contaminated the lungs, as it was not found in lungs in which blood was removed by perfusion. The fifth form was not identified; however, no antibodies to isozymes AA, E, or M were utilized. In addition, no epoxide substrates were tested (53).

The nomenclature is not clear in the kidney. In this tissue, glutathione S-transferase B was identified on the basis of isoelectric point (pI), substrate specificity, and antigenicity (54). In addition, other laboratories have reported that renal cytosol has activity toward DCNB, PNBC, and EPNP (55), indicating that isozymes A, C, and E may also be present. Thus, the precise identity of all of the renal isozymes is unresolved.

That the glutathione S-transferases are distinct proteins and not merely the result of proteolytic cleavage or interconversion has been indicated by several studies. The amino acid difference index is a measure of the similarity of proteins. An index of zero indicates identity; a value of 100 indicates a lack of homology. A comparison of glutathione S-transferases A and C (which are cross-reactive) yields an index of 2.2; AA and B, 7.4; A and B, 9.5; B and C, 8.8; and AA and C, 12.0 (56). Furthermore, with isozyme B the mRNA specific for each subunit is differentially induced by phenobarbital, implying that the subunits are encoded by different genes and are not proteolytic cleavage products (44). Partial proteolytic digestion of isozyme C and AA yielded many different bands and very few similar ones, again stressing the differences (57). However, although the genes encoding the subunits of isozyme B are distinct, great similarity exists between the subunits. Both peptide mapping (45) and cloning of the cDNA (58) indicate up to 90% homology.

The catalytic mechanism of the glutathione S-transferases is at present unclear. Several possible mechanisms exist, including a general base catalysis, nucleophilic attack of the thiol of glutathione on a carbon or nitrogen of the electrophilic substrate, lowering of the pK_a of the thiol of glutathione to increase its nucleophilicity, simple proximity effects, or activation of the electrophilic center. Support for the general base catalysis is primarily from two lines of evidence. First, all of the conjugation reactions catalyzed by the glutathione S-transferases tend to be spontaneous. By increasing the pH of the reaction mixture, the non-catalyzed rate can be increased to equal the catalyzed rate. This suggests the function of the enzyme as a base catalytic one. Secondly, the best rate enhancement (or k_{cat} divided by the second-order rate constant for noncatalytic reactions k_2) is not great, about 500 M for isozyme C with CDNB (59). This falls into the range which would be reasonably expected for a general base catalysis.

Another reasonable mechanism of catalysis is the nucleophilic attack of the GSH thiol on an electrophilic site of the second substrate in an S_N2 mechanism. Firm support for this mechanism was reported by Keen _et_ _al_. (59) by the use of Hammett plots. The Hammett equation relates the effects of meta- and para-benzene-substituted derivatives to the rate of reaction. It states that the rate of reaction of the derivatives may be related to a parent compound, frequently benzoic acid. Alterations in rate induced by the addition of groups in the meta or para position reflects the ability of the groups to attract or repel electrons through both inductive and resonance effects. A plot of the logarithm of the rate constant versus sigma (a constant which is a characteristic of the substituent) yields a line of slope rho. Reactions with a positive rho are aided by electron withdrawal from the benzene ring (making an S_N2 attack possible) (60). Hammett plots by Keen et al. (59) yielded lines of correlation coefficients of 0.96 and 0.98 with rho values of +1.8 and +1.6 for isozymes B and C, respectively. These data provide excellent support for the S_N2 nucleophilic attack mechanism.

Support for the mechanism of lowering the pK_a of the thiol to make it a more powerful nucleophile also exists. The pK_a of the thiol is 9.66 in water (61). As the solvent pH increases toward this value, rates of reaction in the presence and absence of enzyme become equal. In fact, it is believed that GS^- and not GSH is the active nucleophile in the reaction.

The enzymes may function simply by a proximity effect: bringing the nucleophilic thiol of GSH closer and in the proper orientation to the electrophilic second substrate. Some of the substrate specificity implies this mechanism. For example, almost all of the rat liver isozymes catalyze conjugation of the bulky epoxide benzo[a]pyrene 4,5-oxide with GSH. However, with the epoxide EPNP, which is smaller in size and bears the epoxide ring on the alkyl side chain, only A and E catalyze conjugation. It is possible that with AA, B, and C, the cavity for the electrophilic substrate is so "deep" that a smaller epoxide is not close enough to GSH, so no catalytic enhancement is seen.

Finally, although little evidence exists for the possibility that the glutathione S-transferases increase the electrophilicity of the second substrate, such a mechanism cannot be ruled out.

The one requirement for glutathione S-transferase activity is GSH. Other thiol compounds, as L-cysteine, L-cystine, glutathione disulfide, dithiothreitol, and N,N-diacetyl-L-cystine do not serve as nucleophilic substitutes for GSH (62).

The electrophilic second substrate structure-activity relationships are far less well understood. One requirement appears to be that this substrate be sufficiently hydrophobic to allow its interaction with the presumed hydrophobic cleft(s) of the enzyme. The presence of multiple binding and/or catalytic sites on the isozymes has not facilitated the definition of structure-activity relationships. For example, some substrates can function as both ligands and inhibitors of enzyme activity. With a substrate such as 3,6-dibromosulphothalein, the K_i and K_D for isozyme AA are 430 μM and 200 μM; C, 30 μM and 80 μM; A, 40 μM and 100 μM, respectively. These differences in K_i and K_D suggest the existence of multiple catalytic and binding sites with different affinities (63).

The overlap in substrate specificity has resulted in the decision of the Commission on Biochemical Nomenclature deciding to recognize the glutathione S-transferases as EC 2.5.1.18. Previous listings related nomenclature to specificity for epoxides (EC 4.4.1.7) or for the carbon skeleton of the substrate (EC 2.5.1.12, 13, and 14). As urged by Jakoby et al. (64), it is apparent that the glutathione S-transferases cannot be classified on the basis of the reactive group or carbon skeleton, such as glutathione S-epoxidetransferase, alkenetransferase, etc.

For the present studies, the nonepoxide substrates were chosen to facilitate purification and comparative analysis of the present results with those of former studies. The epoxide substrate, EPNP, was chosen for this same reason. The other epoxide substrates, styrene oxide (STOX), 1,1,1-trichloro-2,3-propene oxide (TCPO), 1,2-epoxy-3-phenoxypropane (EPOP), epichlorohydrin (EPIC), 1-octene oxide (OCTO), and cyclohexene oxide (CHXO) were chosen for several reasons. With STOX, TCPO, EPIC, OCTO, and CHXO previous work by Hayakawa et al. (65) indicated that all of these are substrates for glutathione S-transferases. Additionally, STOX has been utilized in numerous studies as a substrate to quantitate glutathione S-transferase or epoxide hydrolase activity (66-70). In addition, recent evidence suggests that stereospecificity of the glutathione S-transferases exists in the conjugation of GSH and STOX. As a result, both possible positional isomers and both diastereomers at each position are produced, although in different quantities (71-73). In addition, styrene and STOX are widely used industrial chemicals; STOX has been shown to be mutagenic in the Ames test (74). Styrene is found in cigarette smoke (75) and in drinking water (76). In addition, STOX has been shown to be a major product of microsomal oxidation of styrene (77).

TCPO and EPIC were chosen for several reasons. First, both represent epoxidation products which could result from microsomal oxidation of allyl trichloride and allyl chloride (research currently in progress in this laboratory has shown EPIC to result from allyl chloride). Secondly, TCPO is a potent uncompetitive inhibitor of microsomal epoxide hydrolase (78). Thus, its metabolism to a less electrophilic xenobiotic is strongly dependent on the glutathione S-transferases. EPIC is a widely used industrial solvent for resins, gums, cellulose esters, paints, varnishes, nail enamels, surface active agents, insecticides, plasticizers, and pharmaceuticals (79,80). Both have been shown to be highly toxic, teratogenic, mutagenic, carcinogenic, and capable of damaging DNA (81-85).

EPOP is the epoxide derivative of allyl phenyl ether. It is used industrially as a reactive diluent in epoxy-resin systems, as a stablizing agent with some halogenated compounds, and to improve processing and storage stability of synthetic fibers. It has been shown to be toxic, mutagenic, and carcinogenic (86-88).

OCTO was chosen because of its long unsubstituted carbon chain. It is a product of microsomal oxidation of 1-octene (89).

CHXO was chosen because it is an alicyclic epoxide. It is produced by microsomal oxidation of cyclohexene (77). Previous work has shown that like STOX, multiple positional and cis/trans isomers are possible. However, unlike STOX, not all isomers are made; in fact, no cis-isomers are formed with CHXO by glutathione S-transferase activity (90). Although CHXO is a competitive inhibitor of epoxide hydrolase (91), it is not believed to be carcinogenic (92).

Finally, mention should be made of the regulation of the glutathione S-transferases. The half-life of renal glutathione S-transferase B is approximately 2 days (93). Developmentally, the activity of glutathione S-transferase B in the liver of the one-day-old male Sprague-Dawley rat is about 20% of that seen in the adult liver (94). However, it develops faster than other forms; for example, in the first week after birth 67.5% of the activity toward CDNB is immunoprecipitable by antiserum to isozyme B. Neither L-thyroxine or cortisol induces a precocious increase in activity toward CDNB.

Thyroidectomy was shown to increase the level of glutathione S-transferase B from 59.3 ± 13.3 μg/mg of cytosolic protein in control adult animals to 85.8 ± 13.1 μg/mg protein. Administration of L-thyroxine following thyroidectomy reduced the levels back to 62.6 ± 12.8 μg/mg of protein (95). Hypophysectomy also increased the level of isozyme B to 89.9 ± 17.0 μg/mg of protein and administration of L-thyroxine reduced the level to 63.4 ± 14.1 μg/mg of protein (96).

A male-female difference exists in glutathione S-transferase levels. In the male rat, activity toward DCNB is approximately threefold higher than in the female. However, activity toward CDNB is nearly equal in both males and females. Isozyme B accounts for 3.3 ± 0.2% of total hepatic cytosolic protein in males and 4.5 ± 0.2% in females. Hypophysectomy eliminated this sex difference (97).

The influence of testosterone on regulation of male rat liver glutathione S-transferases has also been studied. Orchidectomy of adult male Sprague-Dawley rats resulted in decreases in activity toward CDNB, TPBO, and PNBC to 76, 64, and 70% of control, respectively. However, activity toward DCNB was increased to 137% of control. Activities toward CDNB and PNBC could be returned to control levels by testosterone administration. Activity toward TPBO in the orchidectomized male was increased to control levels by dihydrotestosterone or 5α-androstan-3α,17β-diol, but not by testosterone. Activity toward DCNB was decreased to control levels by testosterone, dihydrotestosterone, 5α-androstan-3β,17β-diol, or 5α-androstan-3α,17β-diol (98).

In the kidney of adult male Sprague-Dawley rats, hypophysectomy resulted in significant increases in activity toward DCNB and PNBC (activity was more than doubled) without significant effects on activity toward EPNP or methyl iodide. Administration of L-thyroxine prevented this hypophysectomy-induced increase in activity toward DCNB. The magnitude of the increase in activity toward DCNB was much greater than that seen in the liver after hypophysectomy (55).

All of these results indicate that in males levels of glutathione S-transferase B and possibly E are maintained at high levels by testosterone, although L-thyroxine may have a suppressive effect on B. Activities toward CDNB are nearly equal in both sexes, whereas activities toward DCNB are significantly higher in males. This implies higher levels of isozymes A and C in the male. Testosterone has a more pronounced affect on maintaining isozyme C, in that castration lowered activity toward TPBO and PNBC. The effects of testosterone on isozyme A indicate a suppression (castration elevated activity toward DCNB but not TPBO). Unfortunately, little is known of the effect of thyroid status and hypophyseal activity on isozymes other than B.

Inasmuch as it has been reported that blood testosterone levels in male F344 rats decline from 1.8 ng/ml (5 months old) to 0.6 ng/ml (12 months old) to less than 0.1 ng/ml (29 months old), an affect of aging on glutathione S-transferases can reasonably be expected (99). However, because of the influence of hormonal factors on these enzymes, animals with evidence of testicular or pituitary tumors were excluded from this project.

Thus, distinct isozymes of the glutathione S-transferases exist. They are composed of two subunits and some isozymes may share one common subunit. These subunits have multiple binding and catalytic sites. The levels of the isozymes are regulated by L-thyroxine, testosterone (and some of its reduced metabolites), and perhaps other hormones. During the process of aging, hormonal status is altered; thus regulation of individual isozymes also may be altered. The results of these alterations would be age-related changes in xenobiotic metabolizing activities of tissues where the glutathione S-transferases exist. The biological consequences might be a function of changes in other biotransformation enzymes, repair enzymes, and the nature of the xenobiotic itself. It is reasonable that increased susceptibility to the toxic, mutagenic, and carcinogenic effects of xenobiotics might occur.

It is the goal of this dissertation to elucidate age-related changes in hepatic and pulmonary cytosolic glutathione S-transferases toward epoxide and nonepoxide substrates in male and female F344 rats. In addition, changes in levels of activity or enzymes in male hepatic and pulmonary cytosol are further explored to explain the enzymic basis for changes.

CHAPTER 1
HEPATIC AND PULMONARY CYTOSOLIC METABOLISM OF EPOXIDES: EFFECTS OF AGING ON CONJUGATION WITH GLUTATHIONE

Introduction

Epoxides are reactive electrophiles which may result from the microsomal oxidation of the unsaturated carbon-carbon bond. They have been shown to be intermediates in the metabolism of aromatic hydrocarbons (100), including polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene (101). In addition, epoxides have been shown to result from the microsomal oxidation of arylalkyl compounds such as styrene (77), alkyl compounds such as octene (89), alicyclic compounds such as cyclohexene (77), and halogenated aliphatic compounds such as allyl chloride (M. S. Kachole and K. C. Leibman, personal communication).

Two enzyme systems exist in the cell for the metabolism of epoxides: microsomal epoxide hydrolase (EC 3.3.2.3) and the cytosolic glutathione S-transferases (EC 2.5.1.18). Although products resulting from the action of either of these two enzyme systems may be more reactive than the original compound, such as the product of epoxide hydrolase action on benzo[*a*]pyrene 7,8-oxide (20,102) or glutathione S-transferase action on dibromoethane (21), actions of these enzymes frequently help decrease binding of the compounds to microsomal proteins or nuclear DNA (24,25,103,104).

Many unsaturated compounds that can be metabolized to epoxides, or the epoxides themselves, are common in the environment. For example, styrene is found in cigarette smoke (75) and in drinking water (76). In addition, it is a widely used industrial chemical (105). The epoxide of allyl chloride, epichlorohydrin (EPIC), is widely used as an industrial solvent for resins, gums, cellulose esters, paints, varnishes, nail enamels, surface active agents, insecticides, plasticizers, and pharmaceuticals (79,80). The epoxide of allyl phenylether, 1,2-epoxy-3-phenoxypropane (EPOP), is used industrially as a reactive diluent in epoxy-resin systems, as a stabilizing agent with some halogenated compounds, and to improve processing and storage stability of synthetic fibers (88).

Many of these epoxides, such as styrene oxide (STOX), (85,106), 1,1,1-trichloropropene oxide (TCPO) and EPIC (81-84), and EPOP (86,87), have been shown to be toxic, mutagenic, capable of binding to DNA, or carcinogenic. Thus, the disposition of epoxides in the ever-increasing elderly population is of interest. Life expectancy in the western world is currently 69 years for males and 77 years for females (107,108). In 1980, it was estimated that 11% of the total population was over 65 years old, this is expected to increase to 15% by the year 2040 (109). Whether epoxide metabolism is impaired with increasing age, with subsequent increases in toxicity, is presently unknown.

Metabolism of STOX by epoxide hydrolase and the glutathione S-transferases has been studied in male CFN rats and C57BL/6J mice 3, 12, and 27 months of age. A significant increase in the metabolic rate of epoxide hydrolase was found in both rats and mice with increasing age. However, cytosolic glutathione S-transferase activity with STOX was reported to be unchanged from 3 to 27 months of age (7). Other epoxides have also been reported to be substrates for the glutathione S-transferases, such as TCPO, EPIC, OCTO, and CHXO (65). Whether glutathione S-transferase metabolism of these epoxides is similarly unaffected by aging is presently unknown. In addition, whether epoxide metabolism by these enzymes in the lung, a site of first exposure to many volatile chemicals, changes with increasing age is also unknown.

In this research, conjugation of six epoxides, representing several classes of compounds, was studied, including the arylalkyl epoxide STOX, the arylalkyl ether epoxide EPOP, the mono- and polyhalogenated aliphatic epoxides EPIC and TCPO, the unsubstituted aliphatic epoxide OCTO, and the alicyclic epoxide CHXO, with liver and lung cytosol prepared from male and female Fischer 344 rats of three age groups. The groups selected were 3-months-old (sexually mature young adults), 12-months-old (about 50% of the mean survival time of 23-29 months) (110,111), and 24-months-old (senescent adults chosen at an age before mortality selected for longer-living group). These studies provide indications as to whether the aging population is at a special risk from potentially toxic epoxides.

Materials and Methods

Chemicals. [^{35}S]Glutathione was purchased from New England Nuclear (Boston, MA) at a specific activity of 40-112 Ci/mmol. Reduced and oxidized glutathione (GSH, GSSG), bovine serum albumin, deuterium oxide, and 0.2% ninhydrin solution in ethanol were all purchased from Sigma Chemical Company (St. Louis, MO). 1-Chloro-2,3-propylene oxide

(EPIC) and 1,1,1-trichloro-2,3-propene oxide (TCPO) were purchased from Aldrich Chemical Company (Milwaukee, WI). 1,2-Epoxy-3-phenoxypropane (EPOP) and 1,2-epoxyethylbenzene (STOX) were purchased from Eastman Kodak Company (Rochester, NY). Pentobarbital sodium (65 mg/ml) was purchased from Butler Chemical Company (Columbus, OH). Sulfosalicylic acid, dimethylsulfoxide, KCl, mono- and dibasic sodium phosphate, disodium ethylenediamine tetraacetic acid (EDTA), 1-butanol, 1-propanol, glacial acetic acid, and Scintiverse I scintillation fluid were purchased from Fisher Scientific Company (Pittsburgh, PA). Cyclohexene oxide was purchased from K & K Laboratories (Plainview, NY), 1-octene oxide from Pfaltz & Bauer (Stamford, CT), methanol for HPLC from Burdick & Jackson Laboratories (Muskegon, MI), and absolute ethanol from Florida Distillers (Lake Alfred, FL). All water used for HPLC was triple-distilled in glass from deionized water; all water for other uses was distilled once in glass from deionized water. All other chemicals were of reagent grade or better. Vacuum distillation of STOX (68-70°C at 10 mm Hg), EPOP (61-63°C at 10 mm Hg), TCPO (40-43°C at 10 mm Hg), and EPIC (60-63°C at 100 mm Hg) was used to purify these chemicals. All other chemicals were used as purchased.

Animals. Young male and female Fischer 344 rats (60-90 days old) designated as COBS® CDF® F344/Crl (F344) were purchased from Charles River Breeding Laboratories (Wilmington, MA). Middle-aged (11-12 months old) and senescent (23-24 months old) male and female F344 rats of the same designation were obtained from the colony of the National Institute on Aging maintained at Charles River Breeding Laboratories. Animals were housed two per cage in hanging-wire basket cages. They were allowed free access to food (Purina Rat Chow) and tap water and were maintained on a 12-hour light-dark cycle (0700-1900 hours). All animals were received without evidence of significant infection or pathology. Body weights and gross external pathology were followed for at least 2 weeks after arrival. Any animal that consistently lost weight or showed signs of illness was not used until it began to gain weight again or signs of illness were not apparent.

Tissue preparation. To prepare liver and lung cytosol that were as free of blood as possible, pentobarbital sodium (65 mg/kg) was injected ip between 0800 and 0900 hours. This dose was found to be without effect on enzyme activities. An incision was then made from the lower abdomen to the upper sternum, the skin and tissue were parted, and the sternum was cut apart with scissors. The left renal artery was severed, the left and right ventricles were cannulated, and 100 ml of 0.9% NaCl solution was slowly perfused; if the tissue still retained a blood-filled appearance, another 50 ml of this solution was perfused. The liver and lungs were immediately excised and placed in ice-cold 10 mM sodium phosphate (pH 7.4) which also contained KCl, 11.5 g/liter. The rat was decapitated and the skull was opened. After the cortex was discarded, the pituitary was examined for evidence of adenomas. The testicles were removed from the scrotum, trimmed of fatty tissue, and weighed. In addition, liver, lung, stomach, and large intestine gross pathology was recorded. Animals with evidence of testicular tumors, the most common neoplastic lesion in F344 male rats (110), and pituitary tumors were excluded from this work. The livers and lungs were cleaned of fatty and connective tissue, blotted dry, and weighed. They were minced with surgical scissors.

Livers were homogenized in 4 volumes and lungs in 3 volumes of ice-cold 100 mM sodium phosphate (pH 7.4). Livers were homogenized by five up-and-down strokes of a ground-glass pestle followed by five up-and-down strokes of a Potter-Elvehjem homogenized fitted with a Teflon pestle. Lungs were homogenized by three 10-second bursts of a Janke & Kunkel IKA-Werk Polytron homogenizer (Tekmar, Cincinnati, OH, set at 50% of maximal speed) each followed by 15-second cooling intervals. This homogenate was then further homogenized by the same procedure used for the liver. The homogenates were centrifuged at 9000g for 20 minutes at 4°C in a Sorvall model RC2 centrifuge (Sorvall, Inc., Norwalk, CT). The floating lipid was removed by aspiration and the supernatant fluid was then centrifuged at 100,000g for 75 minutes at 4°C in a Beckman model L2-65B ultracentrifuge (Beckman Instruments, Fullerton, CA). The supernatant cytosol was poured through a funnel, fitted with a glass-wool plug to remove floating lipid, and collected. This cytosol was used for all subsequent experiments.

Glutathione S-transferase assays. Glutathione S-transferase activity was assayed by a radiometric method modified from Hayakawa et al. (112). In a small tube, 45 µmol of sodium phosphate (pH 7.4), 10 µmol of GSH containing about 0.1 µCi of [^{35}S]GSH, and an amount of enzyme found to give an activity in the linear range with respect to protein (figures 1 and 2), or no enzyme for the non-enzymic values, were mixed in a volume of 450 µl. These tubes were preincubated at 37°C for 3-5 minutes and the reactions were initiated by the addition of the epoxide (STOX, TCPO, or CHXO, 13 µmol; EPOP or EPIC, 11 µmol; OCTO, 10 µmol; except in studies of K_M or V_{max}, in which varying amounts were added). The epoxides were added in ethanol (STOX, EPOP, EPIC, OCTO, and CHXO) or dimethylsulfoxide (TCPO). Tubes were incubated for 2-10 minutes [velocities were linear for 2 minutes (TCPO), 5 minutes (STOX, EPOP, or EPIC), and 10 minutes (OCTO and CHXO), figure 3] at 37°C in a Magni-Whirl (Blue-M Company, Blue Island, IL) shaking water bath. The reaction was stopped by the addition of 50 µl of ice-cold 10% sulfosalicyclic acid solution and placement of the tubes on ice. The tubes were centrifuged for 10 minutes at 200g in an International model CS centrifuge (International Equipment Company, Needham, MA), after which 25 µl of the supernatant fluid was spotted on silica gel G (20 x 20 cm, 250 µm thick; Analtech, Newark, DE) TLC plates. The plates were developed for 4 hours in a solvent system of 1-butanol/1-propanol/ glacial acetic acid/water, 4:1:1:2 (v/v). After drying, the plates were sprayed lightly with 0.2% ninhydrin solution and were heated at 55°C for 20 minutes. Alternatively, a sheet of Kodak SB-5 film (20 x 25 cm, Eastman Kodak Company) could be placed on the plate and developed for 3 days (figure 4). However, inasmuch as the exposed bands on the film corresponded to the ninhydrin-positive bands on the TLC plates, ninhydrin was routinely used to visualize the GSH-epoxide conjugates. The bands corresponding to GSH-epoxide conjugates were scraped into 20-ml polyethylene scintillation vials. Scintiverse I liquid-scintillation fluid (8 ml) was added and the vials were shaken for 10 minutes. The radioactivity was quantitated in a Beckman model LS7000 liquid-scintillation counter by use of the ^{14}C window settings. Enzymic specific activity was calculated from the formula:

$$\frac{\dfrac{\text{enzymic cpm - nonenzymic cpm}}{\text{total cpm in 25 } \mu\text{l}}}{\text{min x mg of protein}} \times 10{,}000 \text{ nmol} = \text{nmol/min/mg of protein}$$

Analysis of metabolites. In order to verify that spots which were scraped from the TLC plates were indeed GSH-epoxide conjugates, several criteria were used. First, when systems containing only buffer plus GSH, buffer plus GSSG, or buffer plus epoxide (but no GSH) were used, no ninhydrin-positive spot corresponding to the R_F values of the GSH-epoxide conjugate was present. However, if the buffer, epoxide, and GSH were present in the incubation, a ninhydrin-positive spot was visible which did not overlap the spots due to GSH or GSSG. The addition of cytosol increased the intensity of this spot from two to six times that seen when active enzyme was not added. When autoradiograms of TLC plates were prepared, radioactivity was found in three bands corresponding to the R_F values of GSH, GSSG, and the GSH-epoxide conjugates. In addition, another band was present in the GSH and was believed to be a radiolabelled impurity. The GSH-epoxide conjugates were isolated by HPLC with two Waters Associates model 6000A pumps, a model 660 solvent programmer, and a 254-nm wavelength absorption detector (Waters Associates, Milford, MA). Samples were injected through a model U6K injector onto a μBondapak-C_{18} column (3.9 mm ID x 30 cm, 10 μm particle size) at a flow rate of 1.0 ml/min. For collection of GSH-epoxide conjugates, isocratic systems of methanol/glacial acetic acid/water, 4:1:95 (EPIC, R_F values of 6:40 and 7:40 minutes; CHXO, 8:50 and 10:00), 10:1:89 (STOX, 11:30 and 13:50; EPOP, 11:55), or 15:1:84 (TCPO, 10:00; OCTO, 12:15) (all proportions v/v) were used. With isocratic solvent systems, the conjugates of STOX, EPIC, and CHXO were each resolved into two peaks which are believed to be different positional and/or stereoisomers. The STOX peaks could be further resolved into multiple peaks by a programmed linear gradient as described by Steele _et al_. (71). No attempt was made to isolate these individual peaks or to isolate individual EPIC or CHXO peaks. However, van Bladeren _et al_. (90) have provided evidence for production of two of four possible diastereomers of GSH-CHXO conjugates from rats given CHXO.

After collection of the GSH-epoxide conjugates from the HPLC effluent, they were dried under a stream of nitrogen gas. The resulting gummy solids were resuspended and lyophilized three times in deuterium oxide in order to remove exchangeable protons. Proton nuclear magnetic resonance (NMR) spectroscopy was performed with each metabolite in deuterium oxide (tetramethylsilane was the standard of 0 ppm chemical shift). All spectra were recorded with a JEOL model JNM-FX 100 NMR spectrometer with a spectral width of 1000 Hz, filter of 500 Hz, and a window of 0.1 Hz. In all cases, 16,384 data points were collected and 32 spectra were accumulated. In the event of an excessively large H_2O or HDO peak, double pulsing was used at 9.0-second intervals; the first pulse was for 68 μsec at 180° and the second was for 34 μsec at 90°. Analysis of metabolites yielded these results [which are similar to results obtained by Pachecka _et al_. (113) for GSH and Watabe _et al_. (114) for GSH-STOX]. GSH 2.37 (q), 2.56 (t), 2.97 (d), 3.82 (t), 3.96 (s), and 4.56 (t); GSH-STOX, 2.15 (q), 2.49 (t), 2.86 (d), 2.96 (d), 3.79 (m), 3.86 (s), 4.40 (t), 4.60 (t), and

7.38 (s); GSH-TCPO, 2.25 (q), 2.45 (d), 2.60 (t), 2.95 (d), 3.79 (t), 3.88 (s), 3.90 (t), and 4.60 (m); GSH-EPOP, 2.10 (q), 2.50 (t), 2.95 (d), 3.80 (m), 3.95 (s), 4.04 (m), 4.20 (d), 4.65 (m), and 6.88 (s) ppm. With GSH-EPIC and GSH-CHXO conjugates, the NMR data yielded no distinct structural information; with GSH-OCTO an amount of conjugate sufficient for analysis could not be collected. The second peak of GSH-STOX conjugate was determined to be composed of several unresolved positional and stereoisomers.

When the metabolites that had been collected from the HPLC effluent were developed on the TLC plates, they migrated to the R_F values corresponding to the ninhydrin-positive and radioactive-containing spots seen only when the incubation contained both GSH and epoxide; these R_F values were: STOX, 0.55; TCPO, 0.62; EPOP, 0.60; EPIC, 0.47; OCTO, 0.61; CHXO, 0.49. The conjugates of STOX, EPIC, and CHXO each developed into fairly broad bands, believed to be due to the presence of multiple isomers. The above R_F values were calculated from the centers of these bands.

Protein measurement. Protein was measured by the method of Lowry *et al*. (115) with bovine serum albumin as a standard.

Statistical analysis and calculations. In order to compare the means for the three age groups of animals, a Statistical Analysis System (SAS Institute, Cary, NC) program was used. A General Linear Models (GLM) procedure, which is a modification of a two-way analysis of variance especially for data sets of unequal size, was employed in the analysis. The means were compared by Duncan's Multirange Test. Significance was assumed at $p<0.05$.

All enzymic rates were calculated with a Digital MINC-II computer (Digital Equipment Corporation, Maynard, MA) by use of programs written in BASIC language. All kinetic constants were calculated on the same computer with a weighted Lineweaver-Burk program written in FORTRAN.

Results

Linearity of the conjugations with respect to protein concentration and time. The conjugations of the epoxides with GSH as functions of the amount of cytosol added and time of the incubation are shown in figures 1, 2 and 3. Figures 1 and 2 show rates of reaction as functions of the amount of liver (1) or lung (2) cytosol from young male rats. In all other age groups and sexes, conjugation of STOX with GSH was similarly followed (data not shown). As a result of these studies, in all subsequent reactions, about 0.5-0.8 mg of cytosolic protein was added to each incubation. In addition, linearity of the reactions with respect to time were studied with liver and lung cytosol obtained from young males. In figure 3, the rates of reaction versus the time of the reaction are shown with liver cytosol. As a result, the reactions were carried out over the times specified above. The enzymatic rates were at least double the nonenzymatic rates (enzymatic/nonenzymatic rates: TCPO, 2.0; EPIC, 2.6; CHXO, 2.9; STOX, 4.3, EPOP, 4.9, OCTO, 5.9). An autoradiogram of a developed TLC plate is shown in figure 4.

Effect of aging on conjugation of epoxides with GSH in hepatic cytosol. The rates of conjugation of six epoxides in young, middle-aged, and senescent male and female hepatic cytosol are shown in table 1. In the male, activities with TCPO and EPIC were 15-20% higher

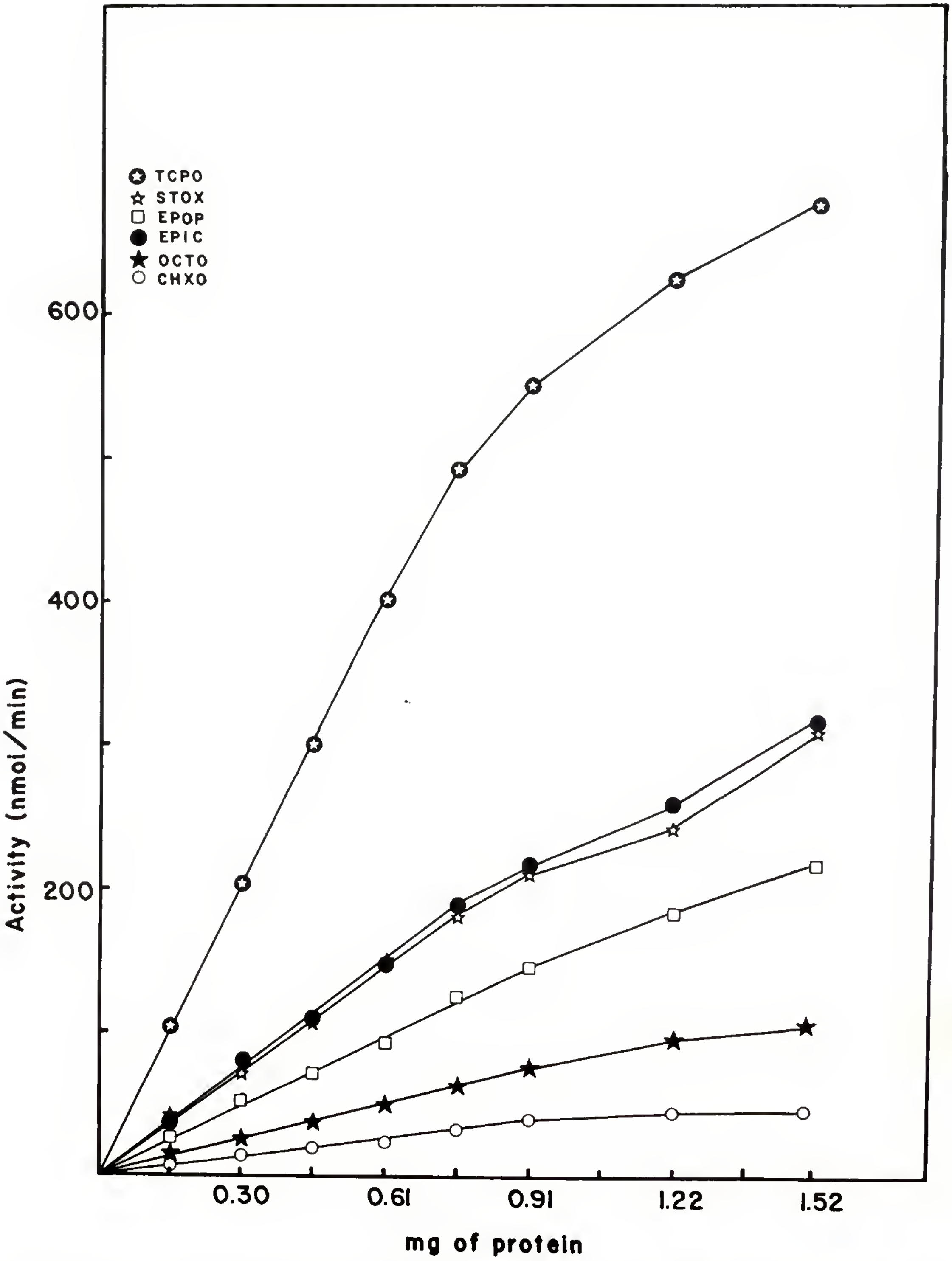


Figure 1. Linearity of the reaction (nmol of conjugate formed per min) as a function of the amount of liver cytosolic protein. This protein is from a young male.

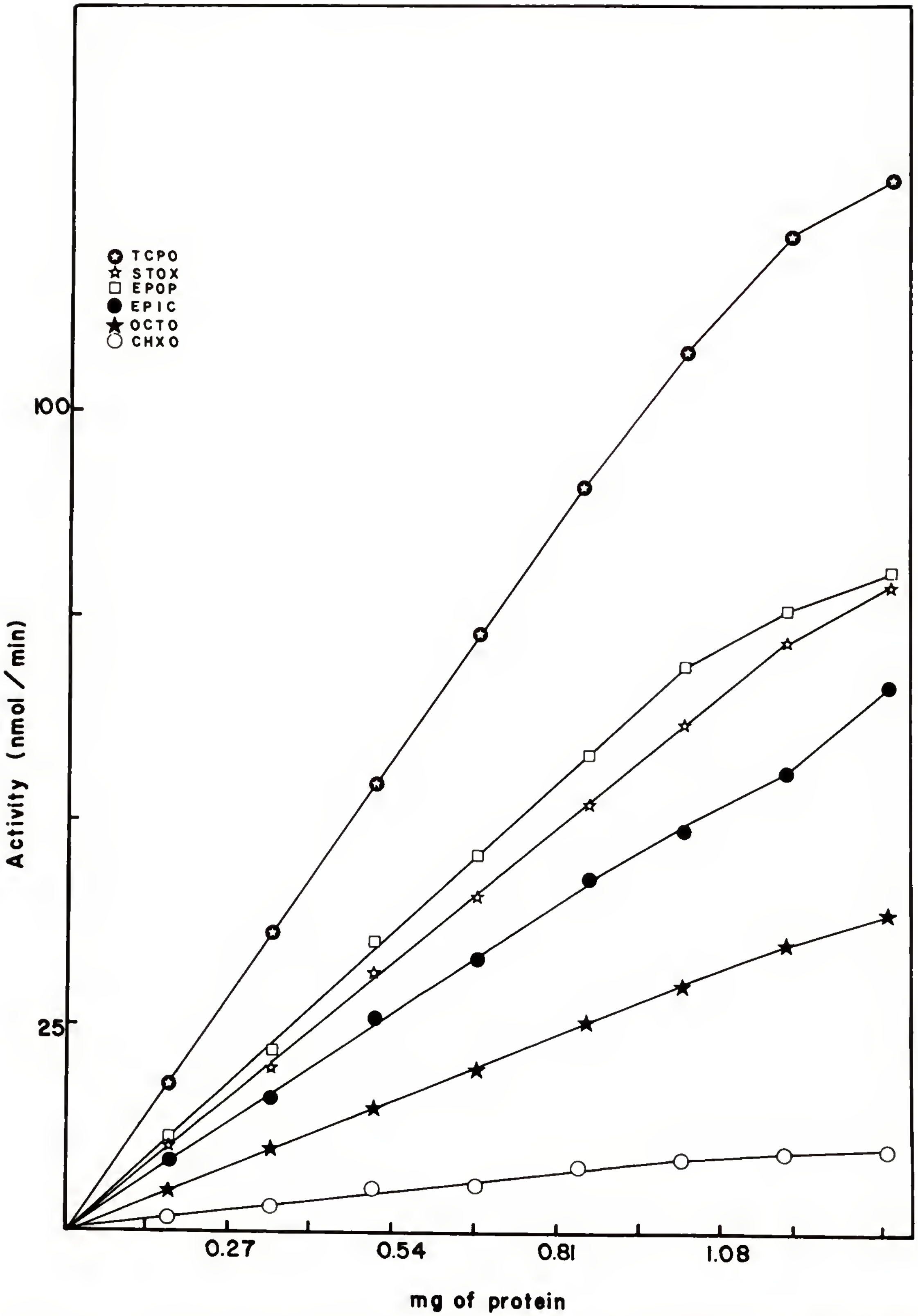


Figure 2. Linearity of the reaction (nmol of conjugate formed per min) as a function of the amount of lung cytosolic protein. This protein is from a young male.

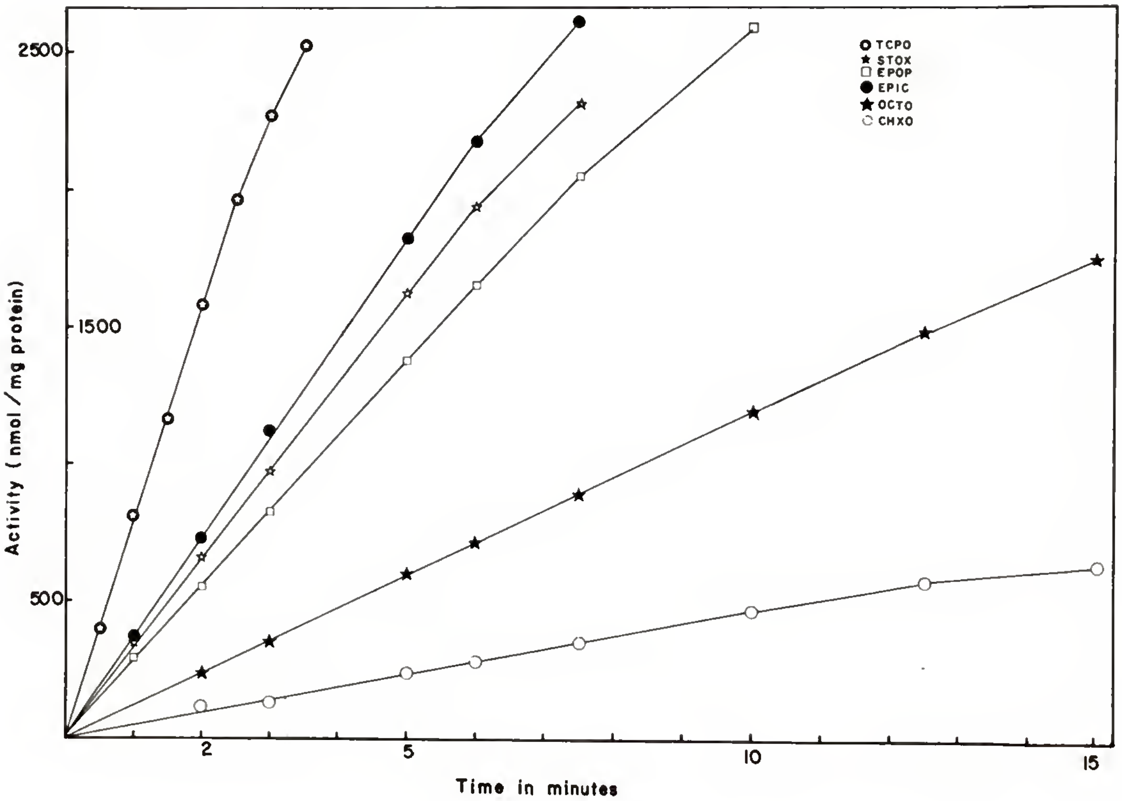


Figure 3. Linearity of the reaction (nmol of conjugate formed per mg of cytosolic protein) as a function of time. Liver cytosolic protein is from a young male.

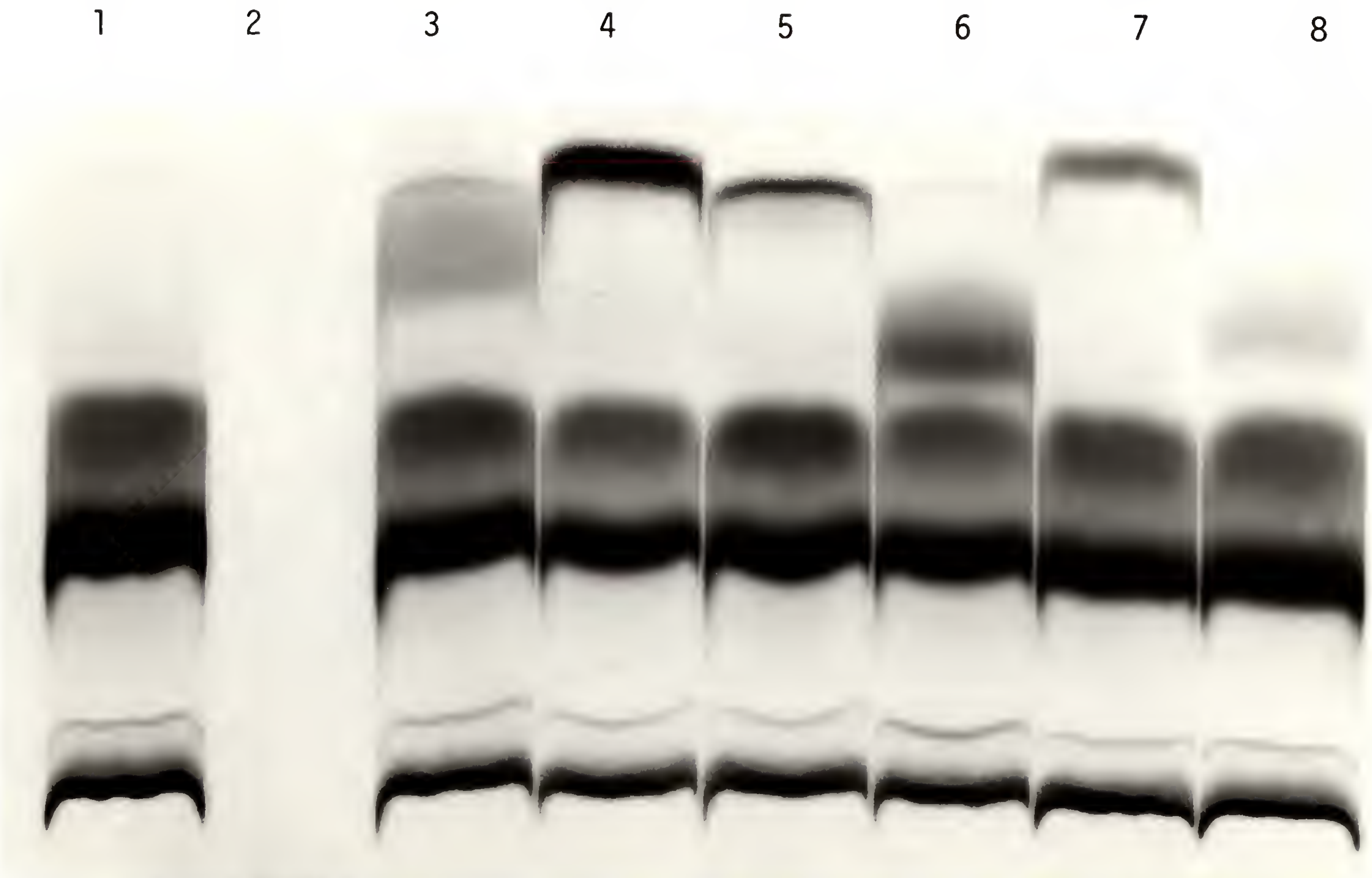


Figure 4. Autoradiogram of a developed TLC plate. Lane 1 contained buffer, GSH (including about 0.1 μCi of [^{35}S]GSH), and liver cytosol. Lane 2 contained buffer, liver cytosol, and STOX. Lanes 3-8 all contained buffer, GSH, and cytosol. In addition, they contained epoxides: Lane 3, STOX; 4, TCPO; 5, EPOP; 6, EPIC; 7, OCTO; and 8, CHXO. Autoradiogram exposure time was 3 days.

in the middle-aged group than in the young group, those with STOX, EPOP, and CHXO were unchanged, and that with OCTO was lower in the middle-aged group. By senescence, activities with all of the substrates were decreased to about 60-75% of those found in the young group. In the female, only activity toward EPOP was significantly different in middle-aged and young animals. By senescence, activities for EPOP and OCTO were significantly lower than in the young group. Generally, the smallest standard errors in the males and the largest in the females were in the young group. This higher variation in old males and young females may reflect the effects of hormonal variations and hormonal influences. For example, with the females, measurements were made without regard to the status of the estrous cycle. Influences of estrogen and testosterone on the levels of glutathione S-transferase isozymes have been indicated (98). In the female, rates of conjugation of STOX and EPIC were unchanged with increasing age; thus by senescence these rates were much higher than those in senescent males. Generally, the magnitudes of the activities were similar in males and females.

Effect of aging on conjugation of epoxides with GSH by pulmonary cytosol. The rates of conjugation of the six epoxides in pulmonary cytosol isolated from young, middle-aged, and senescent groups are also shown in table 1. Activities with cytosol from males were unchanged with increasing age, except those for OCTO and CHXO, which were significantly lower in senescence than in middle age. Activities with cytosol from females were similarly unaffected by increasing age, except for the activity with OCTO, which was significantly decreased by

TABLE 1

SPECIFIC ACTIVITIES OF MALE AND FEMALE HEPATIC AND PULMONARY CYTOSOL

Sex	Organ	Epoxide	3 months old	12 months old	24 months old
Male	Liver	STOX	324 ± 8 (A)	359 ± 21 (A)	206 ± 10 (B)
		TCPO	788 ± 16 (A)	901 ± 47 (B)	606 ± 60 (C)
		EPOP	276 ± 15 (A)	280 ± 22 (A)	190 ± 13 (B)
		EPIC	364 ± 15 (A)	438 ± 26 (B)	254 ± 24 (C)
		OCTO	119 ± 5 (A)	106 ± 4 (B)	75 ± 4 (C)
		CHXO	47 ± 2 (A)	43 ± 4 (A,B)	35 ± 2 (B)
Female	Liver	STOX	340 ± 21 (A)	359 ± 26 (A)	310 ± 17 (A)
		TCPO	695 ± 94 (A,B)	582 ± 32 (A)	773 ± 57 (B)
		EPOP	323 ± 15 (A)	250 ± 7 (B)	222 ± 12 (B)
		EPIC	299 ± 23 (A)	270 ± 20 (A)	319 ± 18 (A)
		OCTO	104 ± 9 (A)	81 ± 8 (A,B)	72 ± 4 (B)
		CHXO	40 ± 11 (A)	28 ± 1 (A)	29 ± 2 (A)

Table 1--extended

Male	Lung	STOX	61 ± 4 (A)	51 ± 7 (A)	45 ± 7 (A)
		TCPO	108 ± 9 (A)	98 ± 16 (A)	85 ± 15 (A)
		EPOP	69 ± 3 (A)	68 ± 8 (A)	53 ± 4 (A)
		EPIC	49 ± 3 (A)	54 ± 6 (A)	64 ± 8 (A)
		OCTO	30 ± 1 (A)	23 ± 2 (B)	23 ± 2 (B)
		CHXO	8.4 ± 0.3 (A)	6.4 ± 0.4 (A,B)	3.9 ± 1.7 (B)
Female	Lung	STOX	64 ± 5 (A)	66 ± 9 (A)	58 ± 3 (A)
		TCPO	94 ± 9 (A)	84 ± 6 (A)	83 ± 12 (A)
		EPOP	70 ± 5 (A)	59 ± 6 (A)	57 ± 3 (A)
		EPIC	54 ± 2 (A)	58 ± 2 (A)	51 ± 2 (A)
		OCTO	29 ± 2 (A)	25 ± 2 (A,B)	20 ± 1 (B)
		CHXO	7.4 ± 1.8 (A)	5.1 ± 1.2 (A)	5.6 ± 1.1 (A)

Data represent means ± SE. Means in a horizontal row with the same letter are not significantly different at $p<0.05$. For males, N = 13 (3 months old), 12 (12 months old), and 11 (24 months old). For females, N = 4 in all cases. Specific activities are expressed as nmol/min/mg of cytosolic protein.

senescence. The increased activities found in hepatic cytosol from middle-aged males (relative to young males) were absent in pulmonary cytosol from either males or females.

Activities per gram of liver and total liver activity. In table 2, glutathione S-transferase activities measured in liver cytosol from male rats are expressed per gram of liver and as total activity in the liver. There was a trend for activity to be greatest in the middle-aged animals and not significantly different between the young and senescent animals. Several exceptions exist, such as with STOX and EPIC, with which the rates per gram of liver were higher in young animals (as were the specific activities in table 1) and with CHXO with which there were no significant changes in any age group (unlike the changes in specific activities). In the female, there was a trend with hepatic cytosol for little significant difference to occur with increasing age (unlike the higher rates shown by middle-age with hepatic cytosol from males), with the exception of TCPO and EPIC, with which activities were greatest in senescent females when expressed per gram of liver or per total liver (data not shown).

In the lung, the trend toward decreased specific activities with increasing age found in both males and females (although significant only for several substrates) was similarly found when the data were expressed per gram of lung. In both sexes, the rates of conjugation per total lung tissue were generally not significantly different with increasing age, although rates of conjugation were highest with EPIC in lungs from old animals (data not shown).

TABLE 2

ACTIVITIES OF MALE HEPATIC CYTOSOL PER GRAM OF LIVER AND TOTAL ACTIVITIES

Epoxide	3 months old	12 months old	24 months old
STOX	16.7 ± 0.5 (A)	21.6 ± 1.5 (B)	12.1 ± 1.0 (C)
	[154 ± 6] (A)	[248 ± 17] (B)	[138 ± 11] (A)
TCPO	40.9 ± 1.7 (A)	54.8 ± 3.1 (B)	36.0 ± 4.2 (A)
	[379 ± 20] (A)	[607 ± 22] (B)	[409 ± 47] (A)
EPOP	14.3 ± 0.8 (A)	17.0 ± 1.5 (B)	11.2 ± 0.9 (A)
	[133 ± 9] (A)	[188 ± 14] (B)	[128 ± 12] (A)
EPIC	18.7 ± 0.6 (A)	26.6 ± 2.0 (B)	14.5 ± 1.3 (C)
	[172 ± 7] (A)	[295 ± 18] (B)	[171 ± 19] (A)
OCTO	6.2 ± 0.3 (A)	6.4 ± 0.3 (A)	4.4 ± 0.2 (B)
	[56 ± 2] (A)	[71 ± 3] (B)	[50 ± 3] (A)
CHXO	2.4 ± 0.1 (A)	2.6 ± 0.3 (A)	2.1 ± 0.2 (A)
	[22 ± 1] (A)	[29 ± 3] (A)	[24 ± 2] (A)

Data represent means ± SE. Means in a horizontal row with the same letter are not significantly different at $p<0.05$. Data without brackets have the units µmol/min/g of liver; those in square brackets represent total activity (µmol/min) per liver. N = 13 (3 months old), 12 (12 months old), and 11 (24 months old).

Age-related changes in protein content and weights of livers and lungs. The changes expressed in table 2 reflect changes in protein content of the cytosol per gram of tissue and changes in organ weights, as shown in table 3. In both sexes, the protein content was significantly increased in the liver by middle-age and remained increased through senescence. Engelmann *et al*. (116) reported that in hepatocytes isolated from female Fischer 344 rats, protein content was significantly increased by 12 months of age (relative to 6-month- old animals) and remained elevated through 30 months of age. Kritchevsky (117) reported that in male Fischer 344 rats, liver weights changed from 9.3 ± 1.0 to 14.5 ± 1.0 to 13.9 ± 0.6 gram in 2, 12, and 24-month-old animals, respectively. Although the protein content per gram of liver increased with increasing age, the rate of protein synthesis has been shown to decline with increasing age (118-120). Thus an overall decrease in protein turnover is implied. In the lung, protein content was not significantly altered in either sex by increasing age. These results are thus in agreement with earlier studies regarding cytosolic protein concentration and liver weights in aging male and female Fischer 344 rats and extend those results to lung tissue from both males and females.

Kinetic constants of K_M and V_{max}. In figure 5 a double-reciprocal plot of reaction rate as a function of concentration of STOX (with GSH held constant at 22 mM) is shown. By the use of this type of plot, the apparent K_M and V_{max} can be calculated and compared between age groups. The data obtained with hepatic and pulmonary cytosol from males and females are summarized in table 4. In both sexes, the V_{max} measured in

TABLE 3

PROTEIN CONTENT AND ORGAN WEIGHTS

	3 months old	12 months old	24 months old
Male			
Protein (mg/g liver)	52 ± 2 (A)	60 ± 1 (B)	58 ± 2 (B)
Liver weight (g)	9.2 ± 0.3 (A)	11.2 ± 0.3 (B)	11.4 ± 0.4 (B)
Protein (mg/g lung)	23 ± 1 (A)	23 ± 2 (A)	27 ± 2 (A)
Lung weight (g)	1.4 ± 0.1 (A)	1.9 ± 0.2 (B)	1.6 ± 0.1 (A,B)
Female			
Protein (mg/g liver)	43 ± 4 (A)	57 ± 2 (B)	54 ± 2 (B)
Liver weight (g)	6.9 ± 0.2 (A,B)	6.3 ± 0.4 (A)	7.7 ± 0.5 (B)
Protein (mg/g lung)	29 ± 1 (A)	28 ± 1 (A)	28 ± 3 (A)
Lung weight (g)	0.9 ± 0.1 (A)	1.0 ± 0.1 (A)	1.4 ± 0.1 (B)

Means in a horizontal row with the same letter are not significantly different at $p<0.05$. For males, N = 17 (3 months old), 16 (12 months old), and 15 (24 months old). For females, N = 4 in all cases. All data are ± SE.

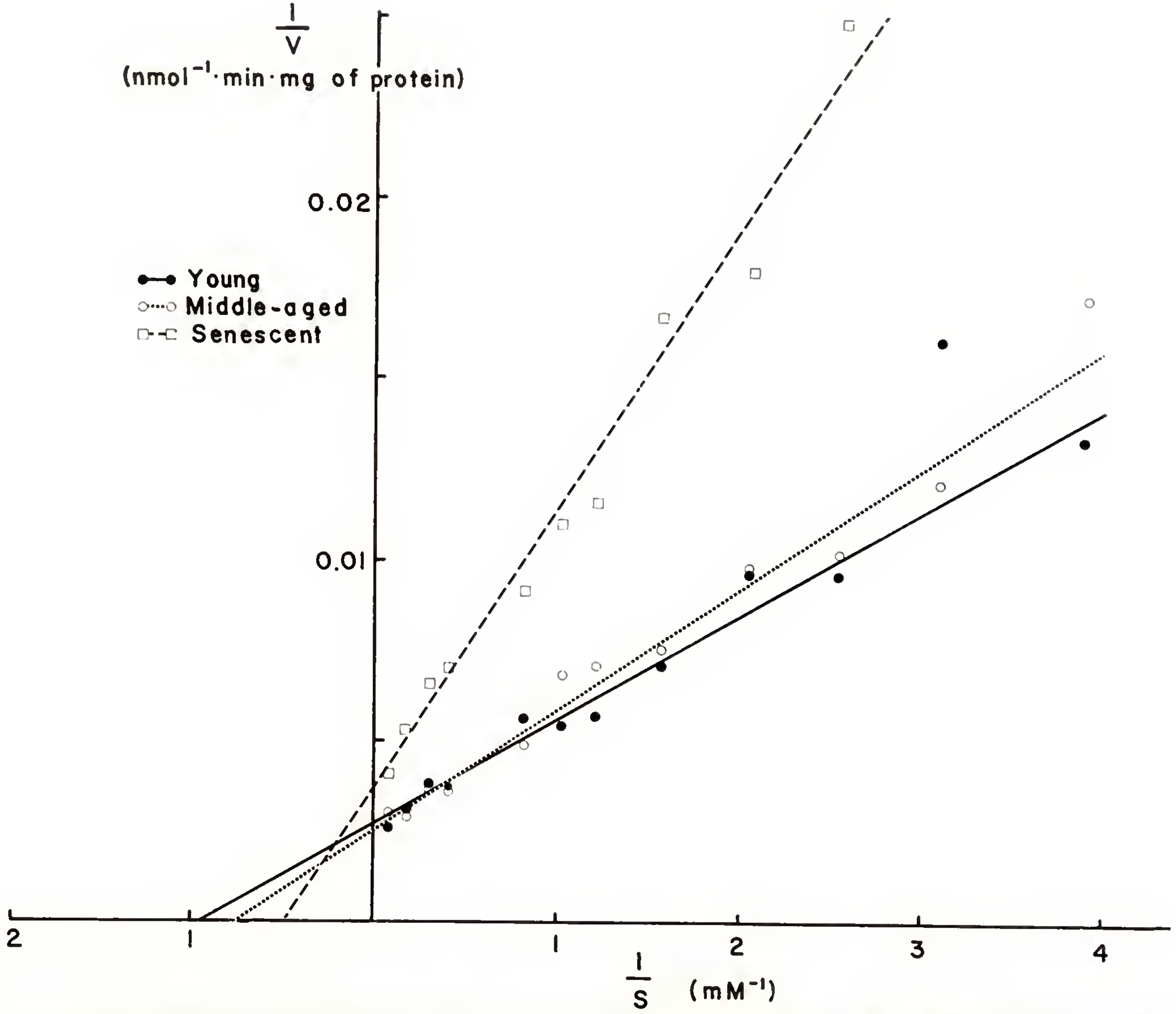


Figure 5. Double-reciprocal plot of the reaction velocity (nmol/min/mg of protein) as a function of the substrate concentration (mM). STOX was the epoxide substrate and concentrations of GSH were 22 mM throughout.

TABLE 4

APPARENT K_M AND V_{max} OF LIVER AND LUNG WITH STOX

Parameter	3 months old	12 months old	24 months old
Male			
K_M (liver)	1.06 ± 0.29 (A)	1.31 ± 0.26 (A,B)	2.13 ± 0.49 (B)
V_{max} (liver)	370 ± 23 (A)	380 ± 20 (A)	256 ± 36 (B)
K_M (lung)	1.03 ± 0.33 (A)	2.41 ± 0.74 (A,B)	2.46 ± 0.26 (B)
V_{max} (lung)	65 ± 6 (A)	58 ± 7 (A)	51 ± 6 (A)
Female			
K_M (liver)	1.50 ± 0.36 (A)	1.52 ± 0.23 (A)	2.06 ± 0.38 (A)
V_{max} (liver)	363 ± 21 (A)	359 ± 12 (A)	257 ± 21 (B)
K_M (lung)	1.55 ± 0.20 (A)	1.55 ± 0.22 (A)	1.20 ± 0.20 (A)
V_{max} (lung)	67 ± 3 (A)	64 ± 2 (A)	54 ± 3 (A)

K_M are expressed as mM and V_{max} as nmol/min/mg of cytosolic protein ± SE. Means with the same letter in a horizontal row are not significantly different at $p<0.05$. GSH concentration was 22 mM throughout. N = 4 for all determinations.

hepatic cytosol is significantly lower in cytosol from senescent than in young or middle-aged animals. The V_{max} values measured with lung cytosol from either sex were not significantly affected by increasing age. In males, the apparent K_M was significantly higher in both liver and lung of senescent animals than in those of young rats. No comparable change was found in the female.

Discussion

The results indicate that during the process of aging tissue-specific, sex-specific, and substrate-specific alterations occur in the metabolism of arylalkyl, alkyl, alicyclic, and halogenated aliphatic epoxides by the cytosolic glutathione S-transferases. Inasmuch as these epoxides are commonly used industrial chemicals that are known to be environmental contaminants, or may result from the microsomal metabolism of commonly used unsaturated compounds, changes in the ability of liver or lung to metabolize them is of toxicologic interest. This study was conducted to discern whether earlier results by Birnbaum and Baird (7), which showed no significant change in STOX metabolism by hepatic glutathione S-transferases during aging of rats, were applicable to other epoxides. The results of this study do not confirm those of Birnbaum and Baird in that age-related changes in STOX metabolism were found in liver cytosol of male rats. Indeed, with liver cytosol of senescent rats, rates of conjugation of all epoxides tested were significantly decreased to 60-75% of the values found with cytosol from young animals. In lung cytosol from males, specific activities were significantly lower in senescent than in young animals only for two of the substrates studied. Thus, in the male, activities in the lung do not appear to be as influenced by the aging process as do those in the liver. However, specific activities in lung cytosol tended to be much lower than those in liver cytosol.

Unlike those measured with the male liver cytosol, specific activities measured with female liver cytosol were significantly decreased only for EPOP and OCTO by senescence. Specific activities in lung cytosol were relatively unaffected by age in the female, similarly to those in the male.

Increases in both hepatic protein concentration and liver weight with increasing age in male rats caused the total hepatic glutathione S-epoxidetransferase activities to rise dramatically between the young and middle-age groups and to fall by senescence only to values equivalent to those in young rats. Similar increases in total hepatic cytosolic protein and generally decreased specific activities resulted in a lack of similar increase in total hepatic activity in middle-aged female rats.

The maximal rates of enzyme activity were significantly decreased by senescence to about 70% of those in young males. In addition, a rise in the apparent K_M was measured. Thus, in the aging male, the cytosolic glutathione S-transferases had both lower affinity for STOX and decreased ability to metabolize it. This tissue would reasonably seem to be at risk from this potentially toxic epoxide. The liver cytosol isolated from females had a similar decline in the maximal rate of STOX metabolism but not in affinity.

In the lung, there were no significant changes in the maximal rates in STOX metabolism in either sex. However, affinity for STOX was significantly decreased in pulmonary cytosol of male but not female rats. This again implies that at low concentrations of STOX, as might be seen with environmental exposure, enzymatic rates of conjugation in pulmonary cytosol from senescent males would be lower than those in cytosol from young males.

The effects of aging on epoxide metabolism suggest several conclusions. First, liver cytosol from male Fischer 344 rats seemed to be greatly affected by aging inasmuch as rates of conjugation of all epoxides were lower by senescence. Thus, this age group may be at the greatest risk of toxicity from exposure to these chemicals. In addition, both the capacity to metabolize and the affinity for STOX were lowest in the liver from senescent males. Second, specific activities and total activities in both sexes were much lower in the lung than in the liver. Thus, exposure to volatile epoxides may present a greater risk to the lung than to the liver. However, the decline in activity noted for all the substrates studied with male liver cytosol was not found in the lung except for two of the substrates. Thus, the potential for damage to the lung during the aging process may be much more dependent upon the substrate than is the potential for damage to the liver in males. Finally, activities in the senescent female liver tended to be significantly decreased to about 70% of young values with several substrates, similarly to the lung (although with different substrates). Thus, similarly to the lung, potential toxicity from exposure to epoxides with increasing age may depend on the substrate.

These results indicate that earlier findings regarding a lack of age-related alterations in male hepatic STOX metabolism may not apply to other epoxides. The difference of rat strain may account for the difference between previously reported results and these results. In addition, these results indicate that the aging process may involve selective alterations in the glutathione S-transferase isozymes. They indicate that certain tissues, such as the liver of aging males, may be more susceptible to influences which alter the levels or activities of these enzymes, therefore making these tissues more susceptible to chemically mediated toxicity. Finally, these results indicate that further characterization of specific changes in individual isozymes of the glutathione S-transferases may help explain the selective age-related changes seen in epoxide metabolism and help predict susceptibility to these toxic chemicals.

CHAPTER 2
THE EFFECTS OF AGING ON HEPATIC AND PULMONARY GLUTATHIONE S-TRANSFERASE ACTIVITIES IN MALE AND FEMALE FISCHER 344 RATS

Introduction

The glutathione S-transferases (EC 2.5.1.18) are a group of cytosolic enzymes that catalyze the conjugation of the cellular nucleophile glutathione (GSH) with electrophiles such as epoxides (65,121,122), aryl compounds (123), nitrate esters (59), alkenes (123), and leukotriene A_4 (124). Many of these electrophilic chemicals, such as epoxides, have been shown to be toxic or carcinogenic (85,102). In addition, these enzymes function as intracellular binding proteins (42), as a Δ^5-3-ketosteroid isomerase (40), and as selenium-independent glutathione peroxidases (37,38). The several isozymes of the glutathione S-transferases have been estimated to compose 5-10% of the total cytosolic protein (63,125).

Two tissues in which the cytosolic glutathione S-transferases are of interest are liver, a quantitatively important organ of drug metabolism, and lung, a site of first exposure to many volatile chemicals which may consequently serve as a site of first metabolic action. The developmental patterns of glutathione S-transferase activities have been well characterized in liver and lung. In the rabbit, James _et al_. (70) reported that the developmental patterns from day 20 of gestation (31-day gestation time) through 4-5 months (sexually mature adult males) were different in liver and lung. In the liver, a very sharp increase was measured between the perinatal specific activities and those at day 6 when quantitated with styrene oxide (STOX). However, in the lung, a much more gradual rise was found. Metabolism of 1,2-dichloro-4-nitrobenzene (DCNB) in the liver rose much more gradually than did activity with STOX; lung followed this similar gradual rise. James and Pheasant (126) followed development of activity in male and female Wistar rats by use of 1,2-epoxy-3-(p-nitrophenoxy)propane, EPNP, a substrate almost exclusively metabolized by glutathione S-transferase isozyme E (123). They found a steady increase in specific activity from day 5 through day 45, with male values being higher than those in the female. Hales and Neims (94) followed maturation of isozyme B in Sprague-Dawley rats. In liver cytosol from 1-day-old males, this isozyme accounted for 67.5% of immunoprecipitable activity toward 1-chloro-2,4-dinitrobenzene (CDNB). After the second postnatal week, this isozyme followed a parallel development with other forms to account for about 50% of immunoprecipitable activity toward CDNB in adult males. By adulthood, the levels of isozyme B are significantly higher in liver cytosol from females than in that from males, accounting for 70% of total immunoprecipitable activity toward CDNB (97). Thus, developmental changes indicate that various glutathione S-transferase isozymes follow somewhat different developmental patterns even in the same tissue and may differ markedly in different tissues and sexes.

Changes in the glutathione S-transferase activities from young adults to senescent animals have not been studied as thoroughly. Birnbaum and Baird (7) reported that with liver cytosol isolated from male CFN rats and C57BL/6J mice 3, 12, and 27 months of age, there were no significant age-related alterations in STOX metabolism. Stohs *et al*. (127) reported that in liver and lung cytosol from aging female Swiss-Webster mice, metabolism of CDNB reached maximal specific activities in 9-month-old animals. In the liver, the specific activities at 18 months of age were decreased to levels that were significantly lower than those from the 3-month-old group. However, in the lung, specific activites in 18-month-old animals were significantly higher than in 3-month-old animals. Kitahara *et al*. (128) reported that in livers from male Fischer 344 rats, specific activity with DCNB was decreased at 24 months of age, compared to 4.5 months of age. However, they found no age-related change in CDNB metabolism.

These studies provide evidence for selective age-related, substrate-specific, and tissue-specific changes in hepatic and pulmonary glutathione S-transferases. To further extend these results, work reported here describes results obtained with liver and lung cytosol isolated from male and female Fischer 344 rats of three age groups: 3 months of age (sexually mature young adults), 12 months of age [approximately midway through a 29-month median lifespan (110)], and 24 months of age (chosen to avoid high mortality that might select a longer-living population). By the use of both sexes of the same strain, it is possible to compare age-related changes without potential strain differences. These results of studies on age-related changes in liver and lung specific activities may thus provide evidence for changes in the ability to detoxify potentially toxic chemicals.

Materials and Methods

Chemicals. Reduced glutathione (GSH) and bovine serum albumin were purchased from Sigma Chemical Company (St. Louis, MO), 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), and *p*-nitrobenzyl chloride (PNBC) from Aldrich Chemical Company (Milwaukee, WI), and 1,2-epoxy-3-(*p*-nitrophenoxy)propane from Eastman Kodak Company (Rochester, NY). Pentobarbital sodium (65 mg/ml) was purchased from Butler Chemical Company (Columbus, OH), mono- and dibasic sodium phosphate and KCl from Fisher Scientific Company (Pittsburgh, PA), and absolute ethanol from Florida Distillers (Lake Alfred, FL). All other chemicals were of reagent grade or better. All water was distilled once in glass from deionized water. CDNB and DCNB were recrystallized twice from ethanol-water before use. All other chemicals were used as purchased.

Animals. Young male and female Fischer 344 rats (60-90 days old) designated as COBS® CDF® F344/Crl (F344) were purchased from Charles River Breeding Laboratories (Wilmington, MA). Middle-aged (11-12 months old) and senescent (23-24 months old) male and female F344 rats of the same designation were obtained from the colony of the National Institute on Aging maintained at Charles River Breeding Laboratories. Animals were housed two per cage in hanging-wire basket cages. They were allowed free access to food (Purina Rat Chow) and tap water and were maintained on a 12-hour light-dark cycle (0700-1900 hours). All animals were received without evidence of significant infection or pathology. Body weights and gross external pathology were followed for at least two weeks after arrival. Any animal which consistently lost weight or showed signs of illness was not used until it began to gain weight again or signs of illness were not apparent.

Tissue preparation. To prepare tissue that was as free of blood as possible, an ip injection of pentobarbital sodium (65 mg/kg) was given between 0800 and 0900 hours. This dose was found to be without effect on enzyme activities. An incision was then made from the lower abdomen to the upper sternum, the skin and tissue were parted, and the sternum was cut apart with scissors. The left renal artery was severed, the left and right ventricles were cannulated, and 100 ml of 0.9% NaCl solution was slowly perfused. If the hepatic and pulmonary tissues still retained a blood-filled appearance, another 50 ml of this solution was perfused. The liver and lungs were immediately excised and placed in ice-cold 10 mM sodium phosphate (pH 7.4), which also contained KCl, 11.5 g/liter. The rat was decapitated and the skull was opened. After the cortex was discarded, the pituitary was examined for evidence of adenomas. The testicles were removed from the scrotum, trimmed of fatty tissue, and weighed. In addition, liver, lung, stomach, and large-intestine gross pathology was recorded. Animals with evidence of testicular or pituitary tumors were excluded from this study. The livers and lungs were cleaned of fatty and connective tissue, blotted dry, and weighed. They were minced with surgical scissors.

Livers were homogenized in 4 volumes and lungs in 3 volumes of ice-cold 100 mM sodium phosphate (pH 7.4). Livers were homogenized by five up-and-down strokes of a ground-glass pestle followed by five up-and-down strokes of a Potter-Elvehjem homogenizer fitted with a

Teflon pestle. Lungs were homogenized by three 10-second bursts of a Janke & Kunkel IKA-Werk Polytron homogenizer (Tekmar, Cincinnati, OH, set at 50% of maximal speed) each followed by 15-second cooling intervals. This homogenate was then further homogenized by the same procedure used for the liver. The homogenates were centrifuged at 9000*g* for 20 minutes at 4°C in a Sorvall model RC2 centrifuge (Sorvall, Inc., Norwalk, CT). The floating lipid was removed by aspiration and the supernatant fluid was then centrifuged at 100,000*g* for 75 minutes at 4°C in a Beckman model L2-65B ultracentrifuge (Beckman Instruments, Fullerton, CA). The supernatant cytosol was poured through a funnel, fitted with a glass-wool plug to remove floating lipid, and collected. This cytosol was used for all experiments.

Glutathione S-transferase assays. Glutathione S-transferase activity was assayed by modifications of methods developed by Habig *et al*. (56,123). A Beckman model 5260 dual-beam UV spectrophotometer was used for all readings. Assays were conducted at room temperature. All incubations were conducted in 100 mM sodium phosphate (pH 6.5). CDNB, DCNB, and PNBC were used in 1 mM concentration, whereas EPNP and GSH were used in 5 mM concentration, except in studies of K_M and V_{max}, in which concentrations were varied. Extinction coefficients that were used were assumed to be the same as those published by Habig *et al*. (56,123). All incubation volumes were 1 ml in semimicro quartz cells. Reactions were initiated by the addition of the cytosol and reaction velocities were linear for 2 minutes.

Protein measurement. Protein was measured by the method of Lowry *et al*. (115) with bovine serum albumin as a standard.

Statistical analysis. In order to compare the means of the three age groups of animals, a Statistical Analysis Systems (SAS Institute, Cary, NC) program was used. A General Linear Models (GLM) procedure, which is a modification of a two-way analysis of variance especially for data sets of unequal size, was employed in the analysis. The means were compared by Duncan's Multirange Test. Significance was assumed at $p<0.05$.

All enzymic rates were calculated with a Digital MINC-II computer (Digital Equipment Corporation, Maynard, MA) by use of programs written in BASIC language. All kinetic constants were calculated with the same computer with a weighted Lineweaver-Burk program written in FORTRAN.

Results

Purified isozyme specific activities. The four substrates selected, CDNB, DCNB, PNBC, and EPNP, are differentially metabolized by individual glutathione S-transferase isozymes. The isozyme substrate specificities, taken from work by Habig et al. (56,123), are shown in table 5.

Effect of aging on specific activities of liver and lung cytosol from males. The results of studies with liver and lung cytosol from males of the three age groups are shown in table 6. With both PNBC and EPNP, specific activities in the liver were significantly higher in middle-aged animals than in the young. These rates decreased by senescence to values that were significantly lower than those found in the young group. Specific activities with DCNB were significantly different only between young and senescent groups; no increase in activity by middle age was seen. Specific activities with CDNB were

TABLE 5

SPECIFIC ACTIVITIES OF PURIFIED GLUTATHIONE S-TRANSFERASE ISOZYMES

Substrate	AA	A	B	C	E
CDNB	14	62	11	10	0.01
DCNB	0.008	4.3	0.003	2.0	<0.0001
PNBC	0.09	11.4	0.1	10.2	4.1
EPNP	n.r.	0.1	<0.006	<0.1	6.7

Values are expressed as μmol/min/mg of protein. Values not reported are indicated by n.r. Taken from references 56 and 123.

TABLE 6

SPECIFIC ACTIVITIES OF CYTOSOL FROM MALES

Organ	Substrate	3 months old	12 months old	24 months old
Liver	CDNB	1317 ± 100 (A)	1385 ± 94 (A)	1256 ± 94 (A)
	DCNB	37.7 ± 1.8 (A)	33.3 ± 1.7 (A,B)	32.6 ± 1.8 (B)
	PNBC	268 ± 12 (A)	339 ± 17 (B)	221 ± 20 (C)
	EPNP	92 ± 2 (A)	119 ± 6 (B)	76 ± 8 (C)
Lung	CDNB	115 ± 8 (A)	109 ± 6 (A)	103 ± 7 (A)
	DCNB	4.0 ± 0.2 (A)	3.7 ± 0.6 (A)	2.7 ± 0.3 (B)
	PNBC	22.5 ± 0.4 (A)	21.7 ± 0.5 (A)	18.1 ± 0.6 (B)
	EPNP	23.8 ± 1.2 (A)	17.8 ± 2.0 (B)	16.7 ± 2.2 (B)

Data represent means ± SE. Means in a horizontal row with the same letter are not significantly different at $p<0.05$. N = 17 (3 months old), 16 (12 months old), and 15 (24 months old). Specific activities are expressed as nmol/min/mg of cytosolic protein.

unchanged throughout the lifespan. These results confirm earlier findings by Kitahara _et al_. (128) regarding metabolism of CDNB and DCNB with liver cytosol.

In the lung, a somewhat different pattern of age-related change was seen. Effects of aging on metabolism of CDNB and DCNB were comparable to those seen in the liver. However, with both PNBC and EPNP, the increase in activity found in the liver cytosol of middle-aged animals was absent in lung cytosol. Instead, a pattern of decline in specific activity was measured with the lung cytosol; this decline was significant by middle age with EPNP and by senescence with PNBC. In addition, the ratios of specific activities in liver to those in lung were about 10:1 for most substrates except EPNP, for which the ratio was closer to 4:1-5:1.

Effects of aging on specific activities of liver and lung cytosol from females. The results of studies with liver and lung cytosol from females of three age groups are shown in table 7. With both PNBC and EPNP, specific activities in liver cytosol from the senescent group were lower than those found with the young group, similarly to results with male liver cytosol. However, unlike the results with liver cytosol of males, there were no significant increase in specific activities with liver cytosol from the middle-aged group. There was no significant decrease in DCNB metabolism throughout the lifespan, unlike the significant decrease in liver cytosol of males found by senescence. However, there was a significant increase in CDNB metabolism by senescence, relative to the young and middle-aged groups. This change in specific activity with CDNB by senescence was also unlike results found with liver cytosol of males.

TABLE 7

SPECIFIC ACTIVITIES OF CYTOSOL FROM FEMALES

Organ	Substrate	3 months old	12 months old	24 months old
Liver	CDNB	1012 ± 77 (A)	1191 ± 65 (A)	1507 ± 97 (B)
	DCNB	31.5 ± 0.9 (A)	30.8 ± 2.1 (A)	32.2 ± 1.1 (A)
	PNBC	268 ± 26 (A)	205 ± 25 (A,B)	160 ± 14 (B)
	EPNP	68 ± 2 (A)	78 ± 1 (A)	50 ± 2 (B)
Lung	CDNB	136 ± 12 (A)	134 ± 10 (A)	129 ± 8 (A)
	DCNB	3.7 ± 0.5 (A)	3.1 ± 0.3 (A)	2.6 ± 0.1 (A)
	PNBC	29.2 ± 1.7 (A)	23.6 ± 1.8 (A,B)	21.0 ± 0.9 (B)
	EPNP	17.6 ± 2.3 (A)	13.4 ± 0.7 (A,B)	10.7 ± 0.6 (B)

Data represent means ± SE. Means in a horizontal row with the same letter are not significantly different at $p<0.05$. N = 4 for all determinations. Specific activities are expressed as nmol/min/mg of cytosolic protein.

With lung cytosol from females, the metabolism of both PNBC and EPNP was significantly decreased by senescence, relative to the young group, similarly to lung cytosol of males and liver cytosol of females. These changes were not significant by middle age, however. No age-related change was found in DCNB metabolism, similarly to liver cytosol of females, but unlike lung cytosol of males. In addition, no change during aging was found in CDNB metabolism, as with lung cytosol of males but unlike liver cytosol of females. Similarly to the liver and lung of males, ratios of specific activities of CDNB, DCNB, and PNBC in liver to those in lung were about 10:1 in the female; the ratio of EPNP in the liver to that in the lung was closer to 4:1-5:1.

Kinetic constants of K_M and V_{max}. The Michaelis-Menten kinetic constants K_M and V_{max} were measured at a constant concentration of 5 mM GSH with varying CDNB concentrations. The results measured with liver and lung cytosol are summarized in table 8. Previous work has shown that glutathione S-transferase isozymes A and C have the lowest K_M with CDNB (0.06 and 0.1 mM, respectively) whereas isozymes AA and B have higher K_M values (0.4 and 0.8 mM, respectively) (56,123). The K_M of isozyme E with CDNB has not been measured due to very low specific activities of isozyme E with CDNB (123). The V_{max} is much higher with isozyme A (3000 moles/min/mole of enzyme) than with AA, B, or C [920, 860, and 500 moles/min/mole of enzyme, respectively (56,123)]. The results in table 8 demonstrate that a significant increase in apparent K_M in liver cytosol of males occurred by senescence, when compared with young or middle-aged groups. No corresponding change in apparent K_M was measured with liver cytosol from females or with lung cytosol from

TABLE 8

APPARENT K_M AND V_{max} OF LIVER AND LUNG WITH CDNB

Parameter	3 months old	12 months old	24 months old
Male			
K_M (liver)	0.095 ± 0.007 (A)	0.090 ± 0.011 (A)	0.122 ± 0.006 (B)
V_{max} (liver)	1192 ± 29 (A)	1414 ± 68 (B)	1371 ± 23 (B)
K_M (lung)	0.25 ± 0.021 (A)	0.25 ± 0.090 (A)	0.26 ± 0.050 (A)
V_{max} (lung)	136 ± 4 (A)	165 ± 22 (A)	139 ± 10 (A)
Female			
K_M (liver)	0.096 ± 0.014 (A)	0.103 ± 0.024 (A)	0.129 ± 0.017 (A)
V_{max} (liver)	1094 ± 50 (A)	1388 ± 61 (B)	1128 ± 79 (A)
K_M (lung)	0.134 ± 0.065 (A)	0.267 ± 0.059 (A)	0.220 ± 0.048 (A)
V_{max} (lung)	142 ± 22 (A)	167 ± 14 (A)	152 ± 13 (A)

K_M are expressed as mM and V_{max} as nmol/min/mg of cytosolic protein ± SE. Means with the same letter in a horizontal row are not significantly different at $p<0.05$. GSH concentration was 5 mM throughout. N = 4 for all determinations.

males or females. With the liver cytosol from both males and females, there was a significant increase in V_{max} in the middle-aged groups relative to the young groups. With liver cytosol of males, this value remained elevated through senescence, whereas with liver cytosol of females the values decreased to those found in the young group. There were no other significant age-related changes in K_M or V_{max} with liver or lung tissue from males or females.

Discussion

These data confirm and extend the findings of Kitahara *et al*. (128) regarding age-related changes in male Fischer 344 hepatic metabolism of CDNB and DCNB. The substrate EPNP is metabolized almost exclusively by glutathione S-transferase E (table 5). The metabolism of EPNP thus gives an indication of the status of the level or activity of isozyme E. In the liver cytosol of male rats, there was a significant increase of about 30% from 3 months of age to 12 months of age. By 24 months of age, specific activities had significantly decreased to about 20% below those in the young group. Thus, isozyme E was increased and then decreased, relative to the young group. With lung cytosol from males and liver and lung cytosol from females, specific activities were decreased by senescence to 70%, 74%, and 61%, respectively, of those found in the young groups. The specific activities by middle age were either unchanged (liver and lung cytosol from females) or significantly lower (lung cytosol from males). Thus, the patterns of change of isozyme E specific activities were both tissue-specific (liver versus lung cytosol of males) and sex-specific (liver cytosol of males versus females). These data also indicate that the specific activities of EPNP metabolism in liver and lung from young males tended to be about 35% higher than those from the corresponding female groups, confirming earlier results (126). Thus, although specific activities in males declined by senescence, they remained higher than those in the female. Isozyme E has been shown to be important in the metabolism of arylalkyl epoxides, such as EPNP; consequently, male liver and lung may be better protected from potential toxic or carcinogenic effects of these compounds.

The substrate DCNB is metabolized predominantly by isozymes A and C (table 5). Although specific activities with isozyme A are about double those with isozyme C, it is believed that there is a greater amount of isozyme C in the liver of uninduced young males (63), so these two isozymes almost equally account for overall rates of DCNB metabolism. It is possible that unchanged specific activities with DCNB could represent an increase in one isozyme with a corresponding decrease in the other. In liver and lung cytosol from male rats, specific activities with DCNB were unchanged in young and middle-aged groups, but were significantly decreased in the senescent groups to 86% and 68% of values found in the young groups, respectively. With females, no change in DCNB metabolism with increasing age was found with liver or lung cytosol. Thus, age-related changes are also substrate-specific, as with metabolism of EPNP and DCNB in liver cytosol of males or females, and therefore isozyme-specific.

The substrate PNBC is metabolized by isozymes A and C equally well and also by isozyme E, although only about 50% as rapidly (table 5). Together with that of EPNP and DCNB, its metabolism aids in further characterization of isozyme alterations. With liver cytosol of males, specific activities in the middle-aged groups were increased 26% above those of young rats. The rates decreased by senescence to 82% of those in young rats. These results agree with the changes found in EPNP and DCNB metabolism by senescence and indicate an overall decline in the A-C-E isozyme group, although an increase in isozyme A or C by senescence with a corresponding decrease in the other form is possible. In lung cytosol of males, no similar increase in PNBC metabolism was found by middle age. However, by senescence, specific activities were significantly decreased to 80% of those in young rats, similarly to those with EPNP and DCNB. Thus, lung isozyme specific activities did not undergo an increase in middle age in males, as with liver. These data indicate that the increase with isozyme E found in liver of middle-aged males did not occur with lung of this group. Instead, a pattern of decline of the A-C-E isozyme group in the lung prevailed throughout the lifespan of the animal, with maximal values in the early portion of the animal's life.

Specific activities with PNBC measured in liver and lung cytosol of female rats followed a pattern of change similar to that found in lung cytosol of males; namely, there was no change through middle age but a significant decrease to 60% (liver) and 80% (lung) of activities in young rats by senescence. In both tissues, a decrease in the A-C-E isozyme group occurred. These decreased activities were significantly different by middle age from those in the young group when measured with some substrates (and thus with some isozymes) and by senescence when compared with other substrates, again indicating isozyme-specific changes during aging.

Finally, CDNB is metabolized by isozymes AA, A, B, and C (table 5). The specific activity with isozyme A is about five or six times higher than with other isozymes. CDNB thus indicates overall changes in the levels of these four isozymes, independent of isozyme E changes. With liver and lung cytosol from males and lung cytosol from females, no age-related alterations were found in CDNB metabolism. With liver cytosol from females, a significant increase was found in CDNB metabolism by senescence (relative to specific activities in the young or middle-aged groups). Thus, the overall levels or activities of the AA-A-B-C isozyme group were not significantly affected by increasing age in liver or lung cytosol of males or lung cytosol of females. However, results with DCNB and PNBC indicated that although specific activities with CDNB were not affected with age, the activities or levels of individual isozymes were affected. As the A-C isozyme group showed an overall decline in liver and lung cytosol of males, the results with CDNB indicate that isozymes AA or B may be increased by senescence. In addition, isozyme A or C could be increased with a corresponding decrease in the other isozyme.

The changes in apparent K_M indicate that only liver cytosol from senescent males had a lower affinity for CDNB. Thus, although there were no significant changes in specific activities toward CDNB with liver cytosol of male rats during aging, the decreased affinity indicated that at low substrate concentrations CDNB metabolism would be decreased in this group.

These results indicate that changes in glutathione S-transferase isozyme levels or activities are both tissue-specific and sex-specific.

These enzymes are important in the metabolism of electrophilic chemicals, such as epoxides. Although some epoxides, such as EPNP, are metabolized by isozyme E, another epoxide, benzo[a]pyrene 7,8-oxide, has been shown to also be a substrate for isozymes A, B, and C (25). Thus, age-related alterations in these isozymes may be important in determining the toxicity of electrophilic epoxides. Although the overall capacity and affinity of the isozymes to metabolize CDNB were generally unchanged with increasing age, changes in the specific activities of EPNP, DCNB, and PNBC indicated that isozyme-specific changes did indeed occur. These data support and substantially extend earlier findings (128). They indicate that with certain tissues and sexes, risks of toxic results from exposure to electrophiles are greater with increasing age.

CHAPTER 3
AGING SELECTIVELY ALTERS GLUTATHIONE S-TRANSFERASE ISOZYMES IN LIVER AND LUNG CYTOSOL

Introduction

The glutathione S-transferases are a family of cytosolic enzymes that catalyze the conjugation of the cellular nucleophile glutathione (GSH) with electrophilic compounds such as epoxides (25,121,122), aryl compounds (123), alkenes (123), organic nitrates (59), thiocyanates (129), or Δ^5-3-ketosteroids (40). Several lines of evidence indicate that the individual glutathione S-transferase isozymes are selectively regulated. These include distinct developmental patterns (70,94,130), differential effects of inducers (131-134), and sex differences and the effects of sex hormones (97,98,135,136). Indeed, not only are the isozymes individually regulated, the subunits composing one of them, transferase B, may also be separately controlled (44,137).

These isozymes may function to decrease the toxicity and carcinogenicity of a number of compounds (24,25,103,138-140). Thus, changes in levels or activities during the aging process could increase the susceptibility to toxic or carcinogenic chemicals, as indicated earlier (22). Earlier chapters in this dissertation and work from other laboratories (10,127,128,141) have indicated that tissue-specific, sex-specific, and substrate-specific changes occurred in the glutathione S-transferases during aging. However, no report of quantitative changes in levels or activities of individual isozymes exists.

Thus, the goal of this work was to quantitate age-related alterations in individual glutathione S-transferase isozymes in male Fischer 344 rats. Three age groups were selected: young sexually mature adults (3 months of age), animals near the midpoint of their lifespan (12 months of age), and old animals selected at an age before significant mortality selected for a longer-living population (24 months of age). Two tissues were studied: liver, a quantitatively important organ of drug metabolism, and lung, a site of first exposure to many volatile chemicals. Thus in this work age-related changes in glutathione S-transferase isozymes that help detoxify toxic or carcinogenic chemicals were quantitated.

Materials and Methods

Chemicals. [^{35}S]Glutathione was purchased from New England Nuclear (Boston, MA) at a specific activity of 40-112 Ci/mmol. Reduced glutathione (GSH), dithiothreitol, Tris, bovine serum albumin, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, ovalbumin, and polyethylene glycol 8000 were all purchased from Sigma Chemical Company (St. Louis, MO). Sodium dodecyl sulfate, N,N'-methylenebisacrylamide, acrylamide, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), p-nitrobenzyl chloride (PNBC), 1-chloro-2,3-propylene oxide (epichlorohydrin, EPIC), and 1,1,1-trichloro-2,3-propylene oxide (TCPO) were purchased from Aldrich Chemical Company (Milwaukee, WI), glycine, 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP), 1,2-epoxy-3-phenoxypropane

(EPOP), and 1,2-epoxyethylbenzene (styrene oxide, STOX) from Eastman Kodak Company (Rochester, NY), cyclohexene oxide (CHXO) from K & K Laboratories (Plainview, NY), and 1-octene oxide (OCTO) from Pfaltz and Bauer (Stamford, CT). Pentobarbital sodium (65 mg/ml) was purchased from Butler Chemical Company (Columbus, OH), ammonium peroxydisulfate, N,N,N',N'-tetramethylethylenediamine, ethanolamine, phosphoric acid, ammonium sulfate, KCl, mono- and dibasic sodium phosphate, disodium ethylenediamine tetraacetic acid (EDTA), and glycerol from Fisher Scientific Company (Pittsburgh, PA). Servalyt AG 5-9, Servalyt AG 8-10, and Servalyt AG 9-11 ampholytes were purchased from Serva Fine Biochemicals (Heidelberg, Germany), Pharmalyte 3-10 ampholytes, Sephadex IEF, and Sephadex G-75 gels from Pharmacia Fine Chemicals (Piscataway, NJ), diethylaminoethylcellulose (DE-52) and carboxymethylcellulose (CM-52) from Whatman, Inc. (Clifton, NJ), and Biogel-HT hydroxylapatite gel from Bio-Rad Laboratories (Richmond, CA). Immunodiffusion plates were purchased from Hyland Diagnostics (Deerfield, IL) and Freund's complete and incomplete adjuvants from Gibco Laboratories (Grand Island, NY). All other chemicals were of reagent grade or better.

All water was distilled once in glass from deionized water. CDNB and DCNB were recrystallized twice from ethanol-water before use. Vacuum distillation of STOX (68-70°C at 10 mm Hg), TCPO (40-43°C at 10 mm Hg), EPOP (61-63°C at 10 mm Hg), and EPIC (60-63°C at 100 mm Hg) was used to purify these chemicals. All other chemicals were used as purchased.

Animals. Young male and female Fischer 344 rats (60-90 days old) designated as COBS® CDF® F344/Crl (F344) were purchased from Charles River Breeding Laboratories (Wilmington, MA). Middle-aged (11-12 months old) and senescent (23-24 months old) male and female rats of the same designation were obtained from the colony of the National Institute on Aging maintained at Charles River Breeding Laboratories. Animals were housed two per cage in hanging-wire basket cages. They were allowed free access to food (Purina Rat Chow) and tap water and were maintained on a 12-hour light-dark cycle (0700-1900 hours). All animals were received without evidence of significant infection or pathology. Body weights and gross external pathology were followed for at least 2 weeks after arrival. Any animal that consistently lost weight or showed signs of illness was not used until it began to gain weight again or signs of illness were not apparent.

Male New Zealand White rabbits were purchased from a local breeder at body weights of 1.5 kg. They were housed individually in hanging rabbit cages and were maintained on a 12-hour light-dark cycle (0700-1900 hours). They were allowed free access to food (Purina Rabbit Chow and lettuce) and water at all times.

Tissue preparation. For studies involving column isoelectric focusing (CIEF), assays of individual animal's hepatic and pulmonary cytosolic activity toward epoxide (STOX, TCPO, EPOP, EPIC, OCTO, CHXO, or EPNP) or nonepoxide (CDNB, DCNB, or PNBC) substrates, or immunotitrations, pentobarbital sodium (65 mg/kg) was injected ip between 0800 and 0900 hours. This dose was found to be without effect on enzyme activities. An incision was then made from the lower abdomen to the upper sternum, the skin and tissue were parted, and the sternum was cut apart with scissors. The left renal artery was severed, the left and right ventricles were cannulated, and 100 ml of 0.9% NaCl solution was slowly perfused. If the hepatic or pulmonary tissues still retained a blood-filled appearance, another 50 ml of this solution was perfused. The liver and lungs were immediately excised and placed in ice-cold 10 mM sodium phosphate (pH 7.4) which also contained KCl, 11.5 g/liter. The rat was decapitated and the skull was opened. After the cortex was discarded, the pituitary was examined for evidence of adenomas. The testicles were removed from the scrotum, trimmed of fatty tissue, and weighed. In addition, liver, lung, stomach, and large-intestine gross pathology was recorded. Animals with evidence of testicular or pituitary tumors were excluded from these studies.

For studies involving extensive large-scale purification of hepatic and pulmonary glutathione S-transferases to homogeneity, animals were killed by decapitation. The liver and lungs were excised immediately and placed in ice-cold 100 mM Tris-HCl (pH 7.4).

In all studies, the livers and lungs were cleaned of fatty and connective tissue, blotted dry, and weighed. They were minced with surgical scissors. For studies involving CIEF, assays of individual hepatic and pulmonary cytosolic activities with epoxide or nonepoxide substrates, or immunotitrations, livers were homogenized in 4 volumes and lungs in 3 volumes of ice-cold 100 mM sodium phosphate (pH 7.4). For studies involving extensive large-scale purification of hepatic and pulmonary glutathione S-transferases to homogeneity, livers were homogenized in 4 volumes and lungs in 3 volumes of ice-cold Tris-HCl (pH 8.05) containing 0.1 mM EDTA. For all studies, livers were homogenized by five up-and-down strokes of a ground-glass pestle followed by five up-and-down strokes of a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Lungs were homogenized by three 10-second bursts of a Janke & Kunkel IKA-Werk Polytron homogenizer (Tekmar, Cincinnati, OH, set at 50% of maximal speed) each followed by 15-second cooling intervals. This homogenate was then further homogenized by the same procedure used for the liver. For all experiments, the homogenates were centrifuged at 9000*g* for 20 minutes at 4°C in a Sorvall model RC2 centrifuge (Sorvall, Inc., Norwalk, CT). The floating lipid was removed by aspiration and the supernatant fluid was then centrifuged at 100,000*g* for 75 minutes at 4°C in a Beckman model L2-65B ultracentrifuge (Beckman Instruments, Fullterton, CA). The supernatant cytosol was poured through a funnel, fitted with a glass-wool plug to remove floating lipid, and collected. This cytosol was used for all subsequent experiments and purifications.

Purification procedures. To prepare glutathione S-transferase isozymes that were separated from each other, yet which were not necessarily pure from other cytosolic proteins, the technique of CIEF was used. A modification of methods of Hales *et al*. (54) was used. Sephadex-IEF (4.4 g) was swollen in 1 mM GSH solution which contained 0.1 mM dithiothreitol. This was packed by gravity (2-3 volumes of the solution) into a jacketed 1.6 x 40 cm column. The column was maintained at 2°C by water which was cooled and circulated by a Lauda K-2/R circulator (Brinkmann Instruments, Westbury, NY). After packing was completed, a continuous gradient of 0-30% glycerol was established on the column to stabilize it from electroendosmosis. This gradient also contained 1 mM GSH, 0.1 mM dithiothreitol, and a 2% mixture of ampholytes composed of Pharmalyte 3-10, Servalyt AG 5-9, Servalyt AG 8-10, and Servalyt AG 9-11 in the ratio of 1:1:2:1 (v/v). The cytosol was washed into the gel by adding it directly to the gradient-forming solutions. A 0.45-μm Millipore filter, which had been saturated with the ampholyte solution, was placed atop the packed gel, and acrylamide was polymerized over this. The column was then inverted, another 0.45-μm filter was placed on the gel, and the procedure was repeated. A small piece of nylon mesh was stretched over this end and secured, and the column was re-inverted. Isoelectric focusing was initiated by placing the bottom of the column in a reservoir of 1% phosphoric acid and filling the top reservoir with a solution of 1% ethanolamine. Platinum wire electrodes were immersed in each reservoir (anode electrode into the bottom), and were connected to a Bio-Rad model 500 power supply. After 2 hours of focusing at 100 volts, the column was focused for 50-60 hours at 500 volts. By this time, the current had decreased to nearly zero. The power supply and cooling hoses were disconnected and the column was cooled to -20°C for one hour. At the end of this time, the acrylamide plugs were removed and the semisolid gel was slowly extruded. The gel was cut into equal sections of 0.8 cm. Each section was vigorously mixed with 3 ml of a 30% glycerol solution containing 1 mM GSH. These sections were centrifuged for 5 minutes at 200g in an International model CS centrifuge (International Equipment Corp., Needham, MA). The pH of the supernatant fluids were measured following the rechilling of each section (Radiometer pH meter equipped with a Radiometer model GK 2232C electrode, Radiometer, Copenhagen, Denmark). The gel was washed three more times with 2 ml of 30% glycerol solution. Supernatant enzymic activities with CDNB, DCNB, and PNBC were measured as described previously. Peaks of activity corresponding to glutathione S-transferases E, C, B, and A were pooled.

For extensive large-scale purification to homogeneity of hepatic and pulmonary glutathione S-transferases, 16-25 rats were killed as described above. All steps were performed at 4°C unless otherwise noted. The methods developed earlier by Jakoby (56,121) were adapted. The cytosol was dialyzed for 12 hours against two changes (4 liter each) of 10 mM Tris-HCl (pH 8.05, Buffer A) and was charged onto a column (2.6 x 100 cm) of diethylaminoethylcellulose equilibrated with Buffer A. This column was washed with 3 liter of Buffer A at a flow rate of 60 ml/hour, and fractions of 10 ml were collected. The effluent was monitored for activity with CDNB and fractions with activity were pooled. Next, ammonium sulfate was added to give a concentration of 160 g/liter and was mixed for 2 hours at room temperature. Ammonium hydroxide (1 M) was added as needed to maintain a pH of 7.3-7.5. Then the turbid solution was centrifuged for 30 minutes at 10,000g at 4°C in a Sorvall model RC-5B centrifuge. The supernatant fraction was poured off and the precipitate was discarded. More ammonium sulfate was added to the supernatant fluid to bring the concentration to 660 g/liter. This was again mixed for 2 hours at room temperature and was centrifuged at 10,000g at 4°C for 30 minutes.

Again the supernatant fluid was poured off, but now the pellet was resuspended in 10 mM sodium phosphate (pH 6.75) containing 1 mM GSH (Buffer B). This was dialyzed for 24 hours against 3 changes (4 liter each) of Buffer B. The dialysate was then charged onto a column (2.6 x 100 cm) of carboxymethylcellulose equilibrated with Buffer B. The column was washed with 1 liter of Buffer B after which a 3-liter linear gradient of Buffer B to Buffer B containing 240 mM KCl was developed at a flow rate of 60 ml/hour. Fractions of 8-10 ml were collected and were monitored for conductance and activity with CDNB, DCNB, and PNBC. Based on these activities, the effluent was pooled into fractions containing glutathione S-transferases E plus D, C, B, A, and AA. Each fraction was concentrated to 15-30 ml in an Amicon concentrator (Amicon Corp., Lexington, MA) fitted with an Amicon PM-10 membrane. Each fraction was then dialyzed against 3 changes (2 liter each) of 30% glycerol solution containing 10 mM sodium phosphate (pH 6.75) and 2.5 mM GSH (Buffer C).

Each fraction was then charged onto a column (1.6 x 40 cm) of hydroxylapatite equilibrated with Buffer C. After a wash with 100 ml of Buffer C, a 1-liter linear gradient of Buffer C to Buffer C containing 240 mM KCl was developed at 30 ml/hour. Fractions of 4-5 ml were collected and were monitored for activity with CDNB, DCNB, and PNBC. Based on activities with these substrates, peaks of activity were pooled and were concentrated to 6-12 ml. Each was dialyzed for 24 hours against 3 changes (1 liter each) of 30% glycerol containing 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, and 2.5 mM GSH (Buffer D). These fractions were washed onto a column (1.6 x 70 cm) of Sephadex G-75, equilibrated with Buffer D. This column was washed with the same buffer at a flow rate of 30 ml/hour. Fractions of 4-5 ml were collected and were monitored for activity with CDNB, DCNB, and PNBC. Again, fractions with activity were pooled and concentrated to 8-12 ml.

These fractions were dialyzed against 3 changes (1 liter each) of Buffer C and were charged onto a column (1.6 x 40 cm) of hydroxylapatite equilibrated with Buffer C. After a wash with 100 ml of Buffer C, a 1-liter linear gradient of Buffer C to Buffer C containing 240 mM KCl was developed at a flow rate of 30 ml/hour. Fractions of 4-5 ml were collected. In all cases, a single peak of activity with CDNB, DCNB, and PNBC was noted. The fractions were pooled and concentrated to 6-10 ml. These were dialyzed against 3 changes (2 liter each) of 30% glycerol solution containing 100 mM sodium phosphate (pH 7.4), 0.1 mM EDTA, and 5 mM GSH. It has been reported previously (51) that the purified glutathione S-transferases are stable for extended periods of time if stored frozen in a very similar buffer (containing potassium phosphate instead of sodium phosphate).

Glutathione S-transferase assays. Assays of activity and identification of metabolites were performed as described in chapters 1 and 2 of this dissertation.

Antibody preparation. Rabbits were injected with either purified glutathione S-transferase E, C, B, A, or AA. The initial injection of 500 µg of isozyme was mixed in Freund's complete adjuvant and was injected in 8-10 subcutaneous sites along the back. The first booster injection (given three weeks later) was of 100 µg of isozyme and was in a mixture of Freund's complete and incomplete (1:16, v/v) adjuvants. Again, the injections were made into multiple sites along the back. All subsequent injections were at 3- to 4-week intervals and consisted of 100 µg of isozyme either in Freund's incomplete adjuvant (second booster) or in 100 mM sodium phosphate (pH 7.4). Animals received from 700-1100 µg of isozyme, depending upon the time required to demonstrate active antiserum (2-4.5 months). Trial bleedings from a marginal ear vein were used to collect blood at several time intervals. When immunotitration and immunodiffusion assays indicated the presence of antiserum to the appropriate isozyme, the rabbits were bled by cardiac puncture after pentobarbital anesthesia (65 mg/kg). The blood was allowed to clot and was centrifuged at 2500*g* in a Sorvall model RC2 centrifuge for 20 minutes at 4°C. The antiserum was separated into 2-ml aliquots and was stored at -20°C until used.

Immunotitration assays. The immunotitration of cytosolic or purified glutathione S-transferases was performed by incubating antiserum (up to 100 µliter) with the enzyme at 4°C in a volume of 350 µl containing 35 µmol of sodium phosphate (pH 7.4). Controls were provided by simultaneously incubating the enzyme without antiserum or by incubating the enzyme with antiserum obtained from Dr. William Kem, who raised the rabbit antiserum to a pure sea anemone neurotoxin (*Stoichactus helianthus*). After 12 hours, 50 µliter of a 22% solution of polyethylene glycol 8000 in 100 mM sodium phosphate (pH 7.4) was added to each incubation tube. Then, after 6 hours at 4°C, each tube was centrifuged at 9000*g* for 20 minutes at 4°C [modified from Hales and

Neims (97)]. The supernatant fluids were used for all subsequent assays with epoxide and nonepoxide substrates as described above.

Immunotitration curves were constructed by adding increasing amounts of antiserum and measuring resulting activity. To compensate for nonspecific losses of activity due to the 12 hour incubation or incubation with serum of rabbits not immunized to the glutathione S-transferases, losses of activity due to incubation without antiserum or with serum obtained from rabbits immunized to the sea anemone neurotoxin were subtracted. Generally, the loss of activity due to incubation with the antiserum prepared to the sea anemone neurotoxin was not greater than 10% of the activity lost merely by incubation of the isozyme for 12 hours in phosphate buffer at 4°C.

Ouchterlony immunodiffusion plates. Ouchterlony immunodiffusion assays were used to verify appropriate cross-reactivity of antiserum and isozymes or antisera and cytosol. Normally, 5 μliter of the antisera was placed in the center of the plate with cytosol or various isozymes (5 μliter of each) in surrounding satellite wells at the 12, 2, 5, 7, and 10 o'clock positions. Plates were normally developed for 24-36 hours at room temperature in a TLC tank lined with paper moistened with water. In some cases, it was necessary to chill the plates to 4°C (following the development process) to retard further formation of precipitin lines until the gels were photographed.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Weber and Osborn (142) with 7.5% acrylamide gels. It was necessary to dialyze glycerol from samples [8 hours of dialysis against 10 mM sodium phosphate (pH 7.4) which contained 2.5 mM GSH]. The samples were next dialyzed for 24 hours against a solution of 2% SDS and 5% 2-mercaptoethanol followed by boiling for 5 minutes. Calibration proteins and their molecular weights were trypsinogen (24,000), carbonic anhydrase (29,000) glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000), and bovine serum albumin (66,000).

Protein measurement. Protein was measured by the method of Lowry *et al*. (115) with bovine serum albumin as a standard.

Statistical analysis and calculations. In order to compare the means for the three age groups of animals, a Statistical Analysis System (SAS Institute, Cary, NC) program was used. A General Linear Models (GLM) procedure, which is a modification of a two-way analysis of variance especially for data sets of unequal size, was employed in the analysis. The means were compared by Duncan's Multirange Test. Significance was assumed at $p<0.05$.

All enzymic rates were calculated with a Digital MINC-II computer (Digital Equipment Corporation, Maynard, MA) by use of programs written in BASIC language.

Results

Column isoelectric focusing of liver cytosol. Liver cytosol (4-6 ml) was separated into isozymes B (pI 9.85), A (pI 9.00), C (pI 8.10), and E (pI 7.15) as described above. Isozyme AA activity was not measured because it frequently ended in the polyacrylamide plug at the cathode end of the column, making quantitation difficult. The activities of each column fraction were measured and a typical separation is shown in figure 6. The isozymes were pooled by separating the fractions at the nadirs of activity (between peaks for isozymes B, A, and C and at the first fraction showing activity for isozyme E). Activities measured with STOX, TCPO, EPOP, EPIC, OCTO, and CHXO are shown in figure 7 (isozyme E), 8 (C), 9 (B), and 10 (A). The data are expressed per gram of liver because the isozymes were not free of other cytosolic proteins, thus making expression as specific activities difficult to interpret. The changes in activities per gram of liver, expressed relative to those in the young liver, are shown in table 9. With isozyme E, rates of metabolism of all substrates were significantly increased by an average of 30% in the middle-aged group and were significantly decreased with most substrates by an average of 19% in the senescent group, relative to the young group. With isozyme C, activities were significantly increased in the middle-aged group by an average of 62% and decreased with some substrates by an average of 40% in the senescent group, relative to the young group. With isozyme B, activities with most substrates were significantly increased by an average of 38% in the middle-aged group and decreased by an average of 40% in the senescent group, relative to the young group. With isozyme A, activities with all substrates were unchanged in the young and middle-aged groups but were significantly increased, by an average of 28%, in the senescent group.

Thus, in the liver cytosol from male rats, isozyme E was quantitatively important in the metabolism of these epoxides. In addition, isozyme B was important in the conjugation of the mono- and polyhalogenated alkyl epoxides EPIC and TCPO. Finally, isozyme A

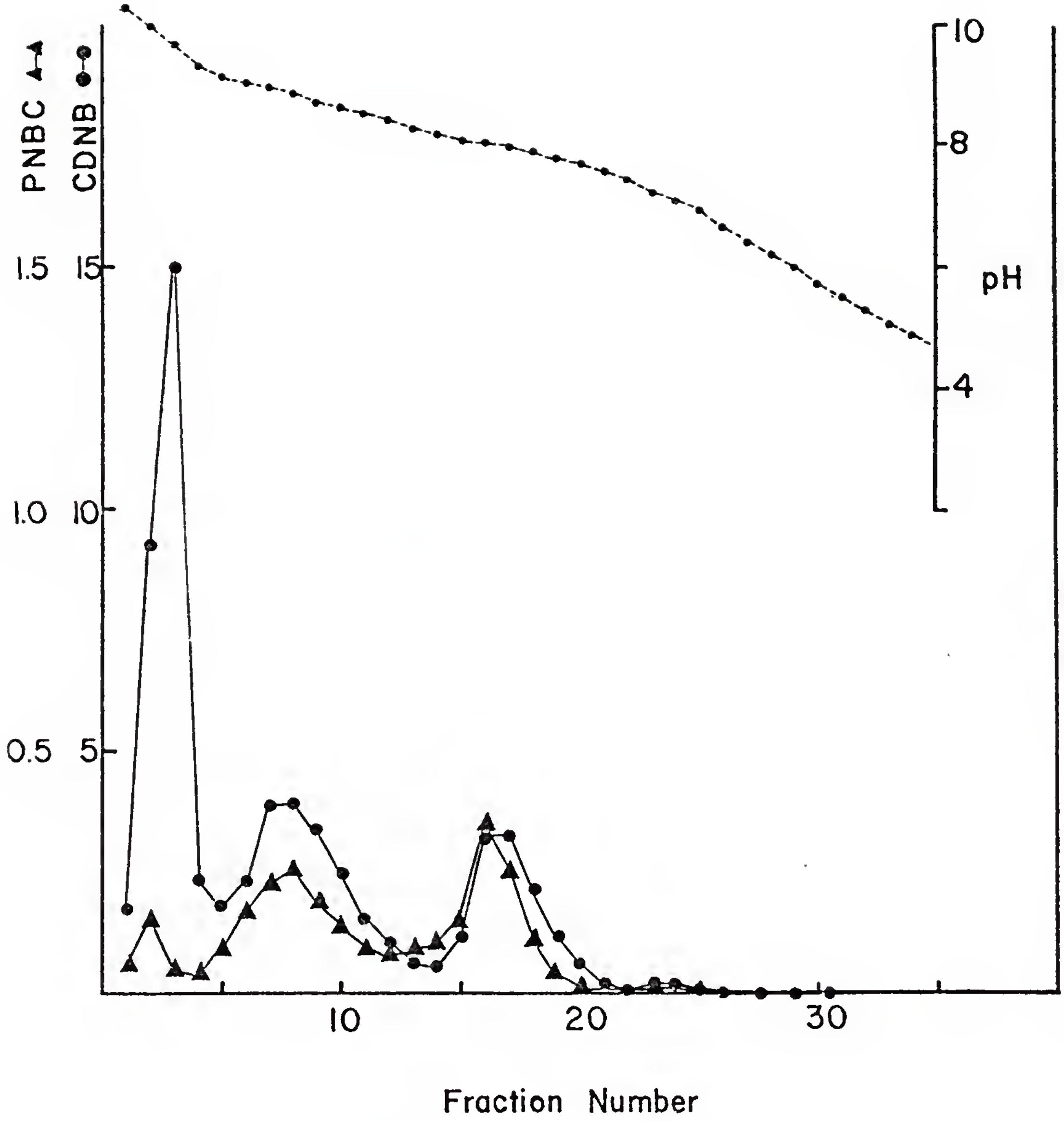


Figure 6. Column isoelectric focusing pattern of liver cytosol from young male rats. Activity is expressed as nmol/min/μliter of column effluent. Activity with PNBC and CDNB are shown. Additionally, the pH gradient is indicated. The four peaks represent (from the left) isozymes B, A, C, and E.

Figure 7. Activities of liver isozyme E prepared by column isoelectric focusing. Means in a substrate group with the same letter are not significantly different at $p<0.05$. Activity is expressed as nmol/min/g liver ± SE. N = 6 (young), 6 (middle-aged), and 5 (senescent). Bars represent young (open), middle-aged (diagonal stripes), and senescent (stipple) values.

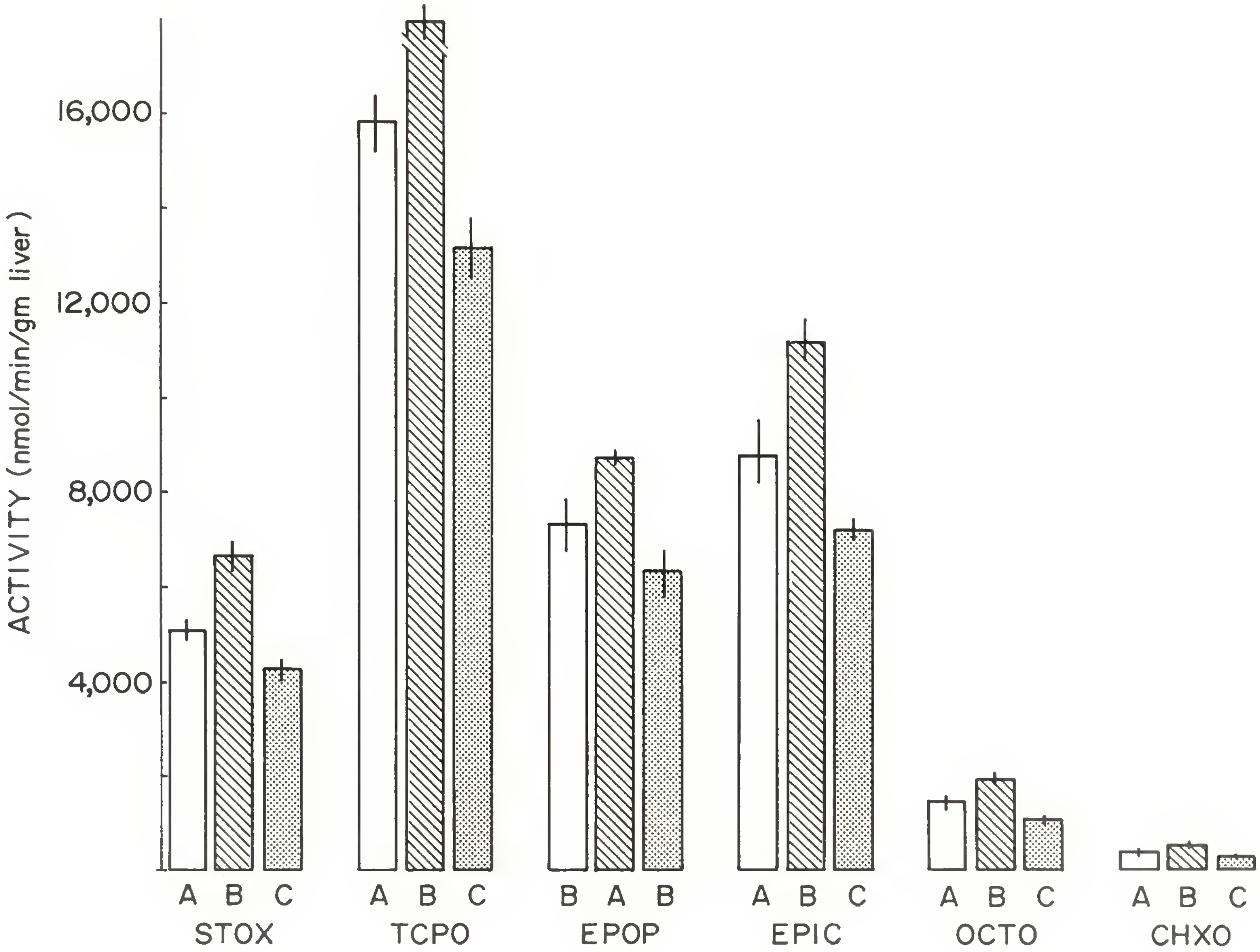

ACTIVITY (nmol/min/gm liver)
16,000
12,000
8,000
4,000
A B C
STOX
A B C
TCPO
B A B
EPOP
A B C
EPIC
A B C
OCTO
A B C
CHXO

Figure 8. Activities of liver isozyme C prepared by column isoelectric focusing. Means in a substrate group with the same letter are not significantly different at $p<0.05$. Activity is expressed as nmol/min/g liver ± SE. N = 6 (young), 6 (middle-aged), and 5 (senescent). Bars represent young (open), middle-aged (diagonal stripes), and senescent (stipple) values.

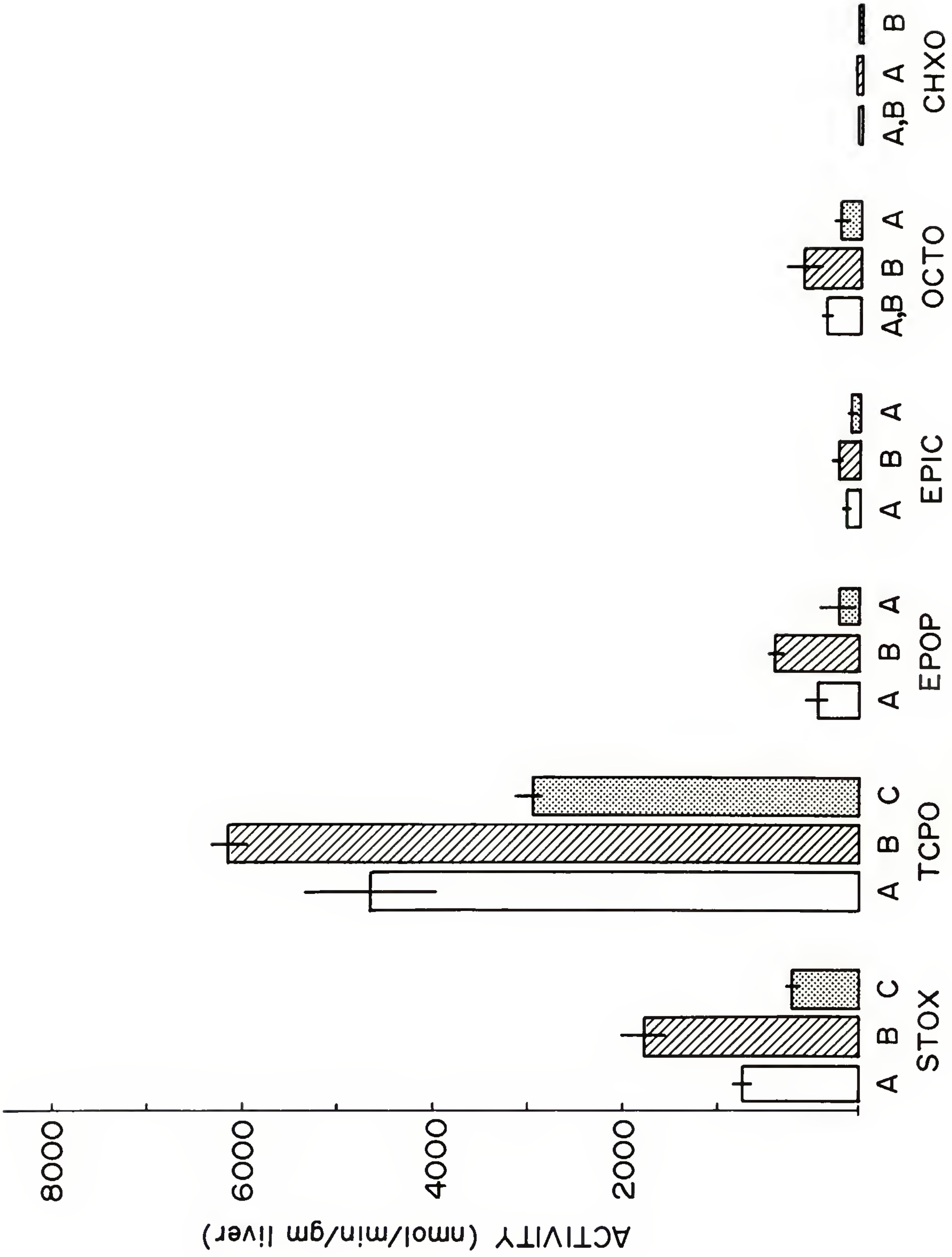

ACTIVITY (nmol/min/gm liver)
8000
6000
4000
2000
A
B
C
STOX
A
B
C
TCPO
A
B
A
EPOP
A
B
A
EPIC
A,B
B
A
OCTO
A,B
A
B
CHXO

Figure 9. Activities of liver isozyme B prepared by column isoelectric focusing. Means in a substrate group with the same letter are not significantly different at $\underline{p} < 0.05$. Activity is expressed as nmol/min/g liver ± SE. N = 6 (young), 6 (middle-aged), and 5 (senescent). Bars represent young (open), middle-aged (diagonal stripes), and senescent (stipple) values.

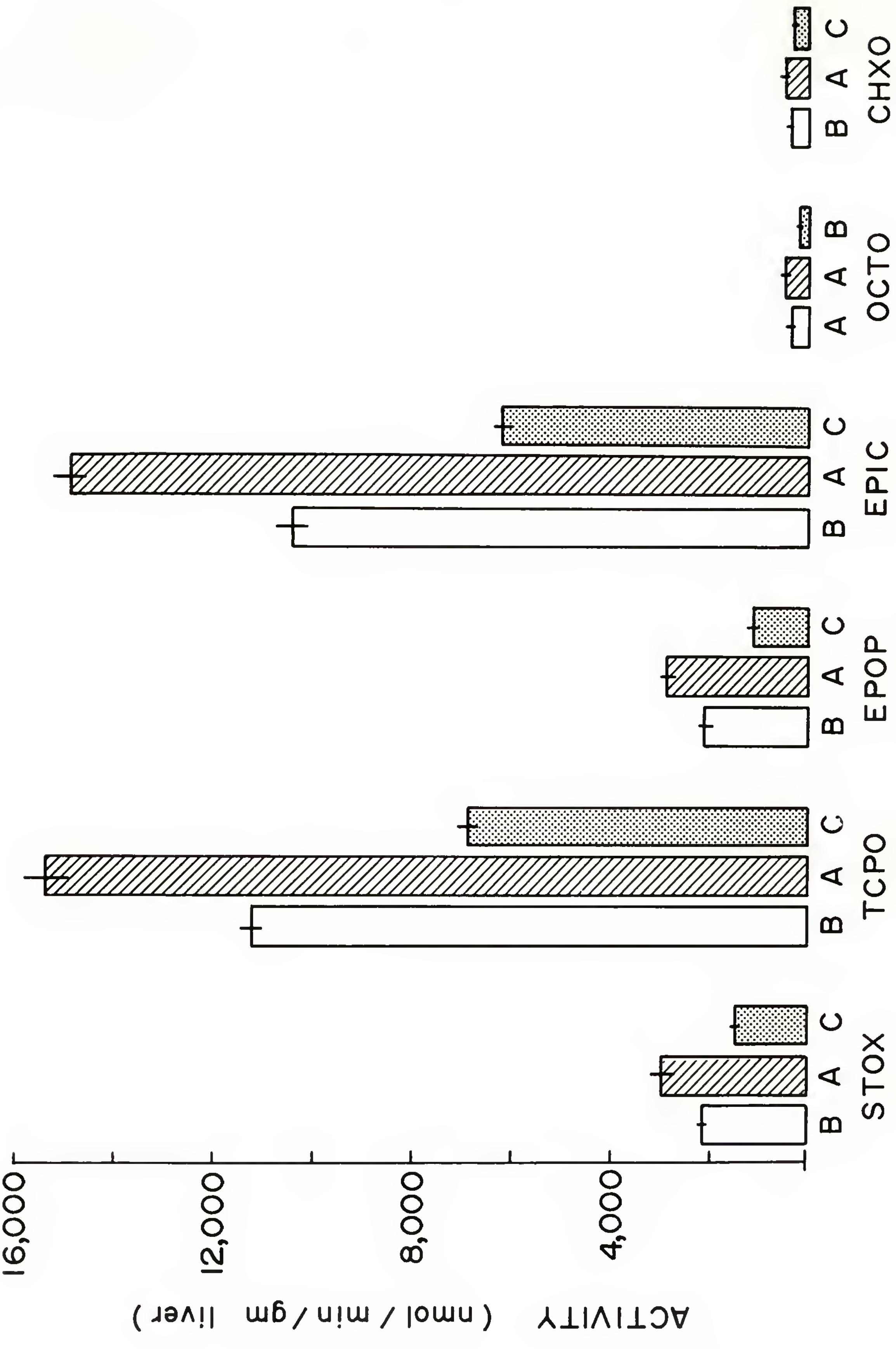
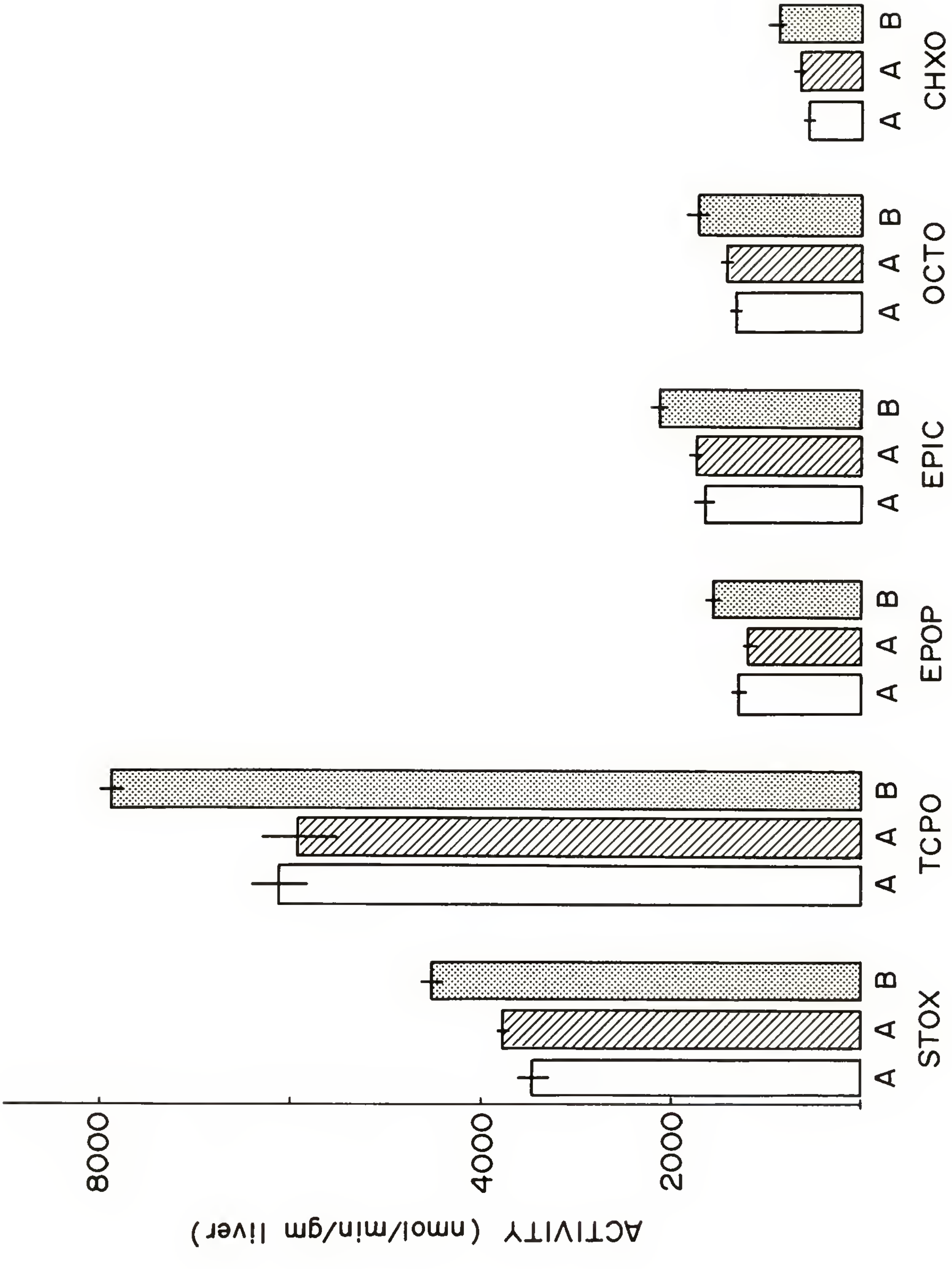

ACTIVITY (nmol / min / gm liver)
16,000
12,000
8,000
4,000
B
A
C
STOX
B
A
C
TCPO
B
A
C
EPOP
B
A
C
EPIC
A
A
B
OCTO
B
A
C
CHXO

Figure 10. Activities of liver isozyme A prepared by column isoelectric focusing. Means in a substrate group with the same letter are not significantly different at $p<0.05$. Activity is expressed as nmol/min/g liver ± SE. N = 6 (young), 6 (middle-aged), and 5 (senescent). Bars represent young (open), middle-aged (diagonal stripes), and senescent (stipple) values.


ACTIVITY (nmol/min/gm liver)
8000
4000
2000
A
A
B
STOX
A
A
B
TCPO
A
A
B
EPOP
A
A
B
EPIC
A
A
B
OCTO
A
A
B
CHXO

appeared to be nearly as important as isozyme E in the metabolism of the unsubstituted aliphatic epoxide OCTO and the alicyclic epoxide CHXO. These data help to explain those reported in chapter 1 regarding age-related changes found in cytosolic epoxide metabolism.

Column isoelectric focusing of lung cytosol. Lung cytosol (about 2 ml) was separated into isozymes B (pI 9.85), A (pI 8.95), C (pI 8.10), and E (pI 7.15) as described above. No activity corresponding in substrate specificity or pI to isozyme AA could be identified. The activities of fractions were measured and a typical separation is shown in figure 11. The fractions were pooled as described above; activities measured with STOX, TCPO, EPOP, EPIC, OCTO, and CHXO are shown in figure 12 (isozyme E), 13 (C), 14 (B), and 15 (A). Data are expressed per gram of lung. Changes in activity, expressed relative to those in the young lung, are shown in table 9. Unlike those of the liver, activities of isozymes E, C, and B did not increase in the middle-aged group. Instead, activities of isozymes E and B were decreased: those of E by an average of 24% (significant with most substrates) and those of B by an average of 27% (significant with some substrates) whereas isozyme C activities were not significantly changed. Similarly to those of liver, activities with isozyme A were unchanged between the young and middle-aged groups, and activities of isozymes E, C, and B were decreased between the young and senescent groups: those of E by an average of 24% (significant with all substrates), those of C by an average of 40% (significant with some substrates), and those of B by an average of 44% (significant with most substrates). The activity of

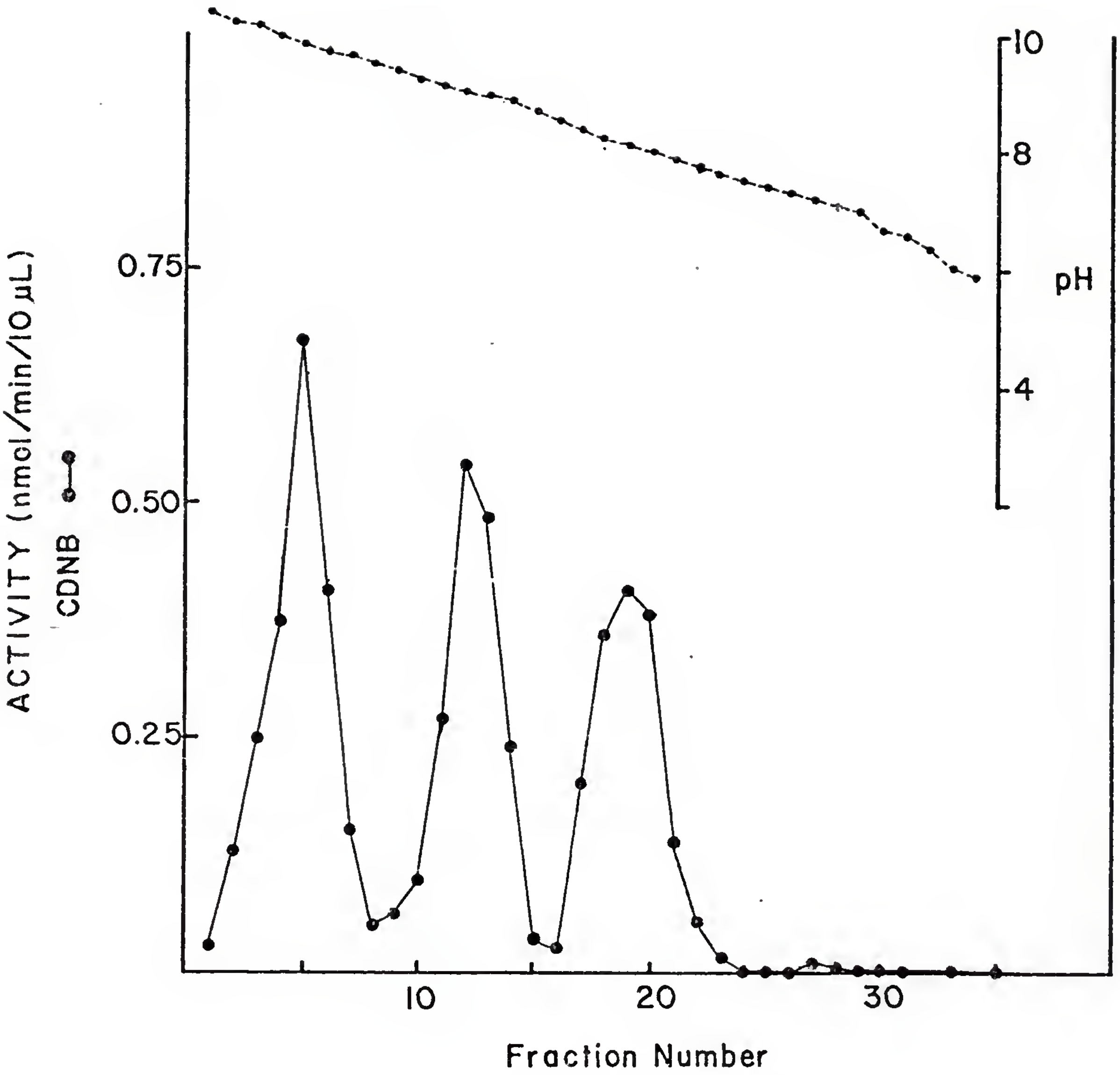


Figure 11. Column isoelectric focusing pattern of lung cytosol from young male rats. Activity is expressed as nmol/min/µliter of column effluent. Activity with CDNB is shown. Additionally, the pH gradient is indicated. The four peaks represent (from the left) isozymes B, A, C, and E.

Figure 12. Activities of lung isozyme E prepared by column isoelectric focusing. Means in a substrate group with the same letter are not significantly different at $\underline{p}<0.05$. Activity is expressed as nmol/min/g lung ± SE. N = 6 (young), 6 (middle-aged), and 5 (senescent). Bars represent young (open), middle-aged (diagonal stripes), and senescent (stipple) values.

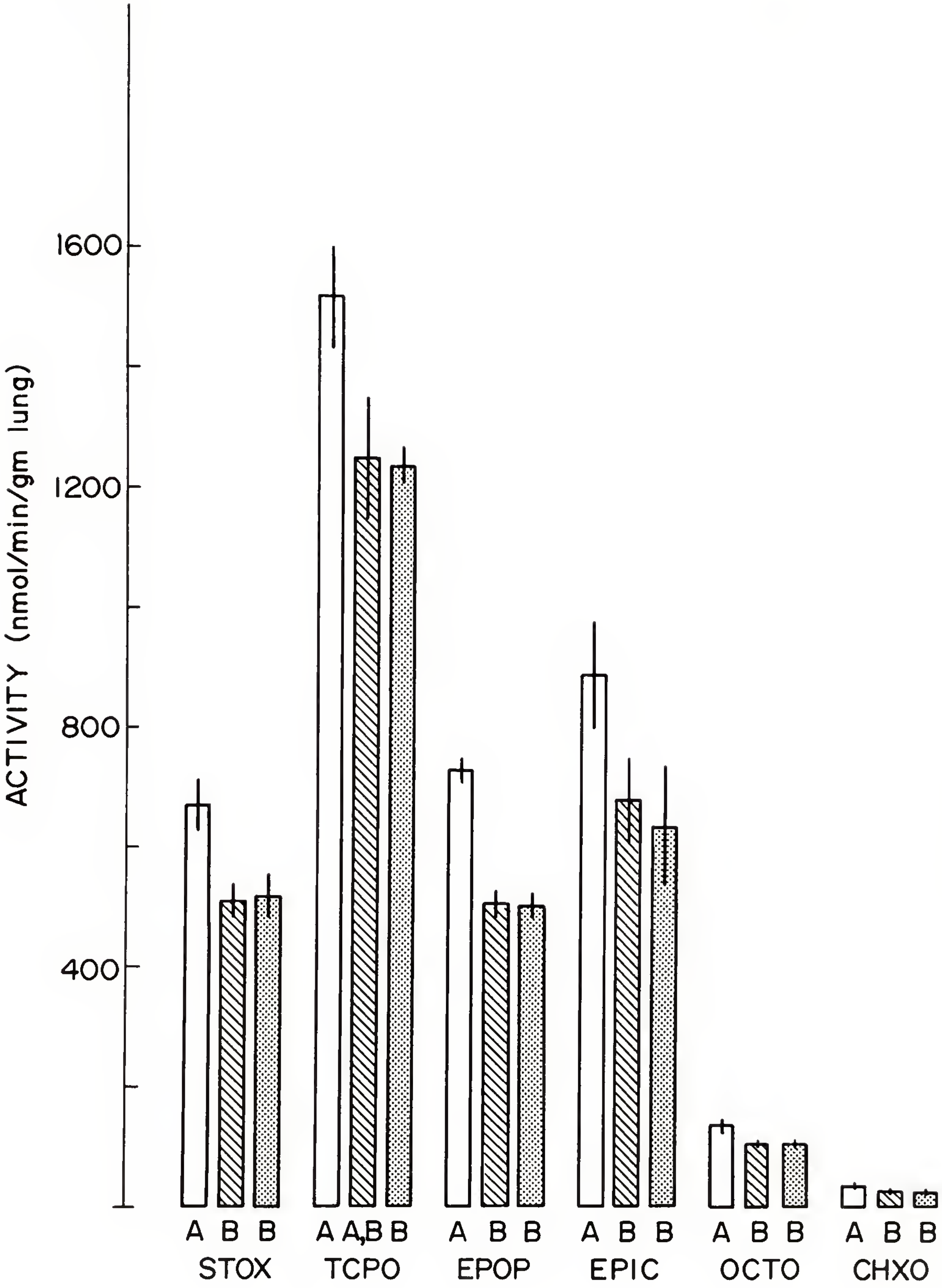

ACTIVITY (nmol/min/gm lung)
1600
1200
800
400
A
B
B
STOX
A
A,B
B
TCPO
A
B
B
EPOP
A
B
B
EPIC
A
B
B
OCTO
A
B
B
CHXO

Figure 13. Activities of lung isozyme C prepared by column isoelectric focusing. Means in a substrate group with the same letter are not significantly different at $p<0.05$. Activity is expressed as nmol/min/g lung ± SE. N = 6 (young), 6 (middle-aged), and 5 (senescent). Bars represent young (open), middle-aged (diagonal stripes), and senescent (stipple) values. Those bars replaced by an asterisk indicate that the activity was not measurable.

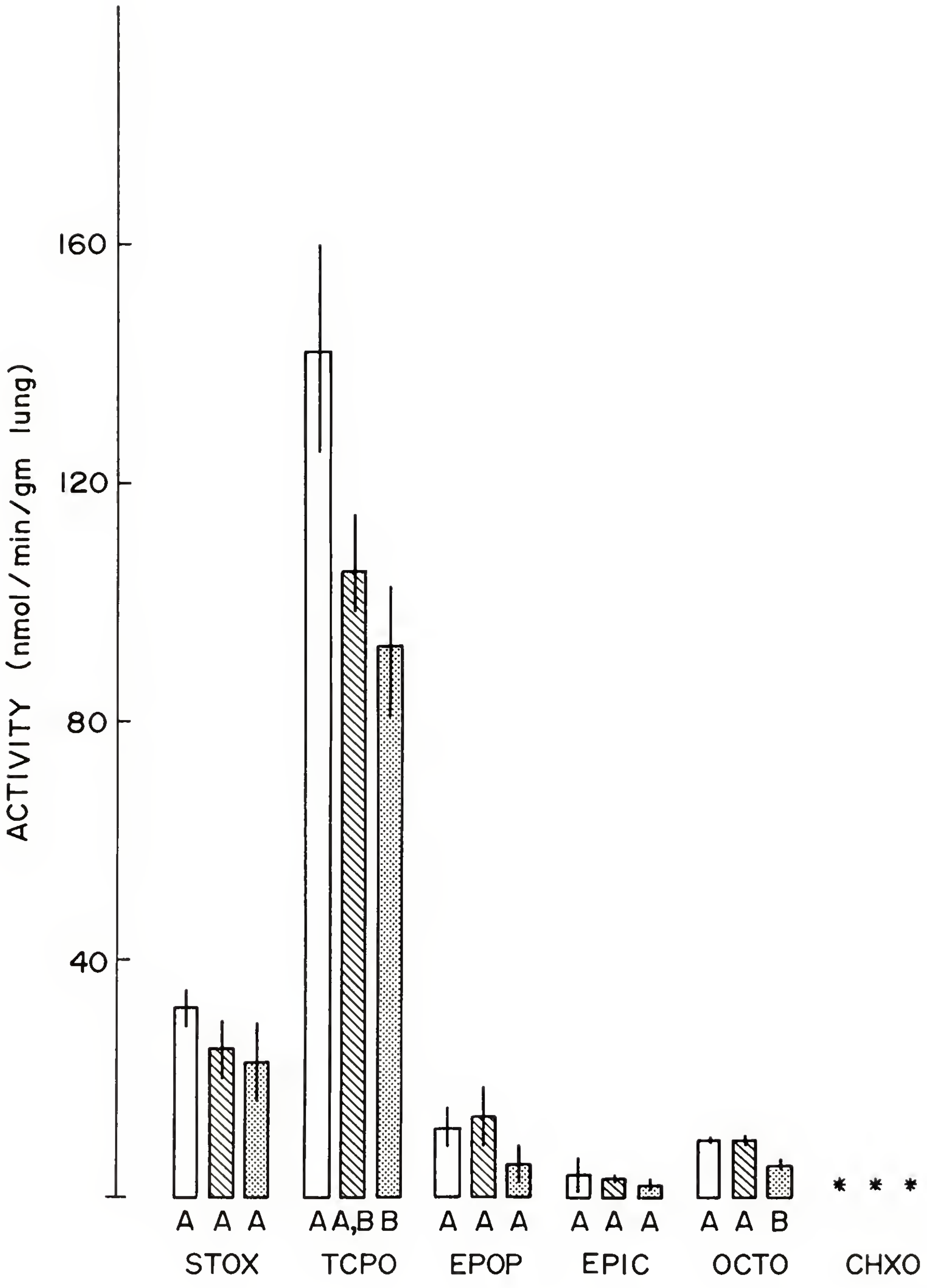

ACTIVITY (nmol / min / gm lung)
160
120
80
40
A A A
STOX
A A,B B
TCPO
A A A
EPOP
A A A
EPIC
A A B
OCTO
* * *
CHXO

Figure 14. Activities of lung isozyme B prepared by column isoelectric focusing. Means in a substrate group with the same letter are not significantly different at $p<0.05$. Activity is expressed as nmol/min/g lung ± SE. N = 6 (young), 6 (middle-aged), and 5 (senescent). Bars represent young (open), middle-aged (diagonal stripes), and senescent (stipple) values. Those bars replaced by an asterisk indicate that the activity was not measurable.

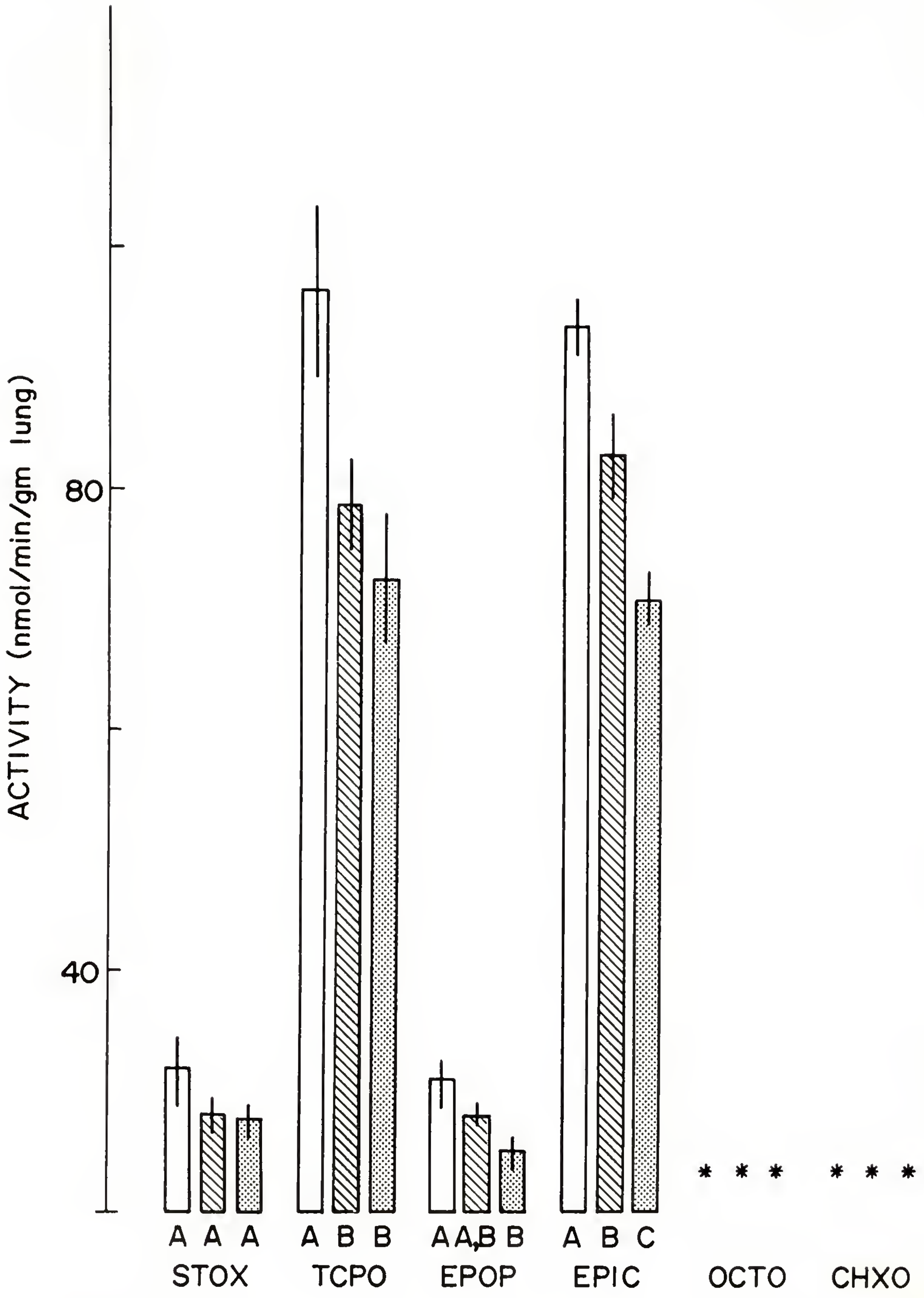

ACTIVITY (nmol/min/gm lung)
80
40
A A A
A B B
A A,B B
A B C
* * *
* * *
STOX
TCPO
EPOP
EPIC
OCTO
CHXO

Figure 15. Activities of lung isozyme A prepared by column isoelectric focusing. Means in a substrate group with the same letter are not significantly different at $\underline{p}<0.05$. Activity is expressed as nmol/min/g lung ± SE. N = 6 (young), 6 (middle-aged), and 5 (senescent). Bars represent young (open), middle-aged (diagonal stripes), and senescent (stipple) values.

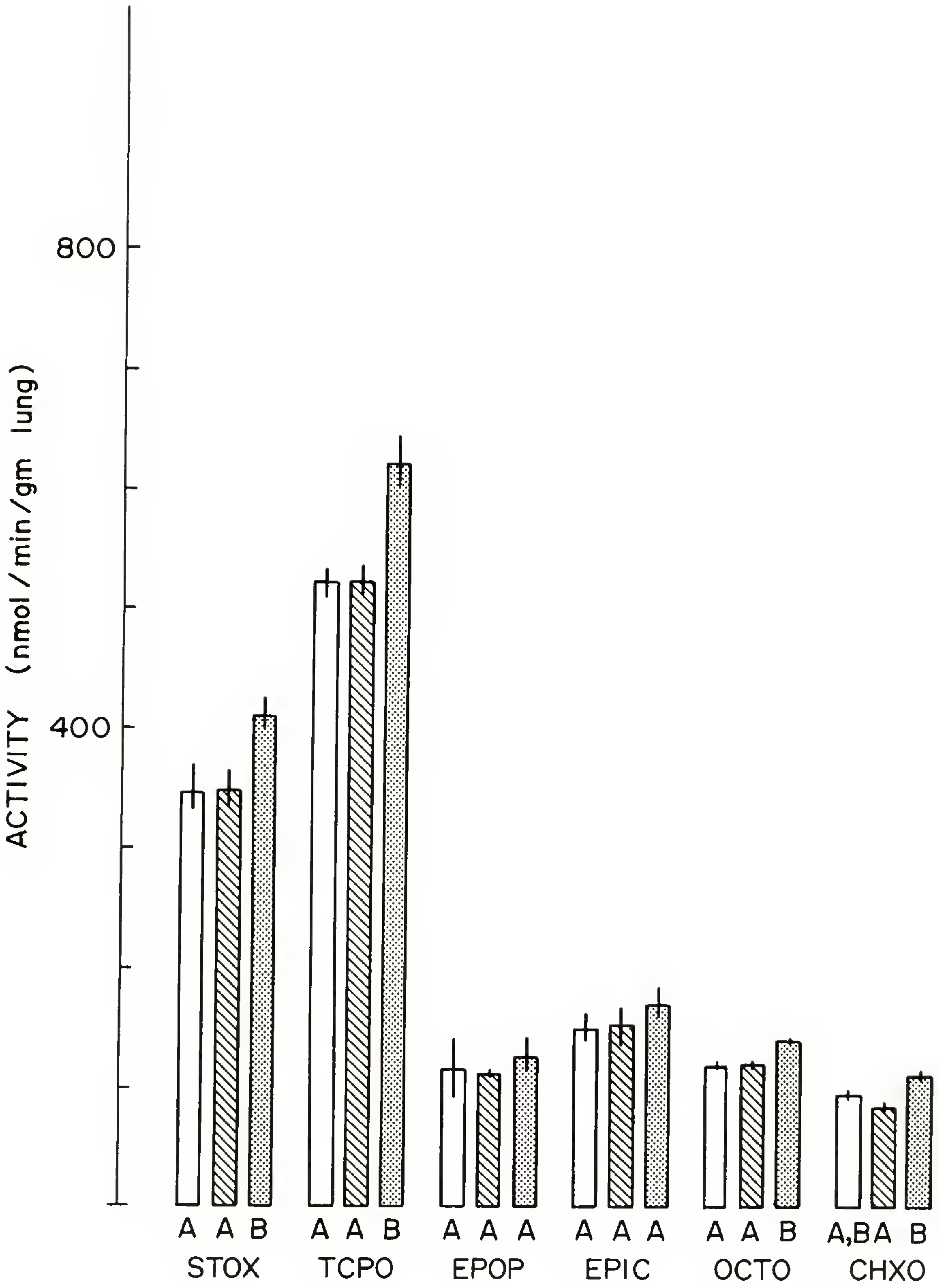

ACTIVITY (nmol / min / gm lung)
800
400
A A B
STOX
A A B
TCPO
A A A
EPOP
A A A
EPIC
A A B
OCTO
A,B A B
CHXO

TABLE 9

AGE-RELATED CHANGES IN HEPATIC AND PULMONARY ISOZYMES SEPARATED BY COLUMN ISOELECTRIC FOCUSING

Tissue	Isozyme	3 months old	12 months old	24 months old
Liver	E	1.00	1.30	0.81
	C	1.00	1.62	0.60
	B	1.00	1.38	0.60
	A	1.00	1.03	1.28
Lung	E	1.00	0.76	0.76
	C	1.00	0.91	0.60
	B	1.00	0.73	0.56
	A	1.00	0.99	1.15

Data are averaged from activities of isozymes obtained by column isoelectric focusing of liver or lung cytosol from each age group. They are expressed relative to the young group; the results for all six epoxide substrates were averaged together. N = 6 (young), 6 (middle-aged), and 5 (senescent).

isozyme A in the senescent lung was increased by an average of 15% (significant with some substrates) above values in young rats.

Similarly to the situation in the liver, isozyme E was important in the metabolism of these epoxides in the lung. In fact, its relative contribution to the overall metabolic rates was proportionally much greater in the lung. Data reported in chapter 2 indicated that although the ratio of specific activities measured with CDNB, DCNB, and PNBC in liver:lung were about 10:1, those with EPNP were closer to 4:1-5:1. Thus, there was a larger contribution to the overall rates of epoxide metabolism by isozyme E in the lung. In addition, isozyme B accounted for a very small contribution to epoxide metabolism in the lung, which prevented measurement of metabolic rates for OCTO and CHXO.

Purification to homogeneity of liver and lung isozymes. Liver and lung isozymes were purified to homogeneity from liver and lung cytosol from each age group. In table 10, results of the purification of isozymes from young liver are shown. The elution pattern of this cytosol from the carboxymethylcellulose column is shown in figure 16. Five peaks of activity with CDNB were separated (fewer peaks with DCNB and PNBC), corresponding to isozymes E plus D (the first to elute from the column), C, B, A, and AA (the final isozyme to elute), as defined by Habig et al. (56, 121, 123). Each isozyme migrated as a single peak of activity toward CDNB and other substrates from the final hydroxylapatite column (not shown).

Isozyme purity. Purity of isozymes was analyzed by SDS-PAGE. The results of SDS-PAGE are shown in figure 17. Each isozyme was separated

TABLE 10

ACTIVITY AND PROTEIN REMAINING AFTER PURIFICATION OF LIVER ISOZYMES

Purification Step	Epoxide	Specific Activity	mg of Protein	Total Activity	% Activity Remaining
Cytosol	STOX	0.354	11,309	4003	100
	TCPO	0.752		8504	100
	EPOP	0.286		3234	100
	EPIC	0.429		4852	100
	OCTO	0.098		1108	100
	CHXO	0.056		633	100
DE-52 Effluent	STOX	0.729	3,019	2201	55
	TCPO	1.905		5751	68
	EPOP	0.605		1826	56
	EPIC	1.139		3439	71
	OCTO	0.209		631	57
	CHXO	0.118		356	56
Ammonium Sulfate Precipitate	STOX	0.946	2,044	1934	48
	TCPO	2.795		5713	67
	EPOP	0.822		1680	52
	EPIC	1.611		3293	68
	OCTO	0.284		580	52
	CHXO	0.163		333	53

Table 10--extended

CM-52 Effluent	STOX	E-4.05	C-0.56	B-0.45	A-5.11	AA-<0.1	740	18
	TCPO	E-9.42	C-2.69	B-2.42	A-7.92	AA-<0.1	1855	22
	EPOP	E-3.71	C-0.23	B-0.47	A-1.74	AA-<0.1	447	14
	EPIC	E-4.22	C-<0.1	B-2.29	A-2.11	AA-<0.1	748	15
	OCTO	E-1.12	C-0.26	B-<0.1	A-2.55	AA-0.86	359	32
	CHXO	E-0.21	C-<0.1	B-<0.1	A-1.55	AA-0.99	212	33
Final Hydroxylapatite Column	STOX	E-13.5	C-1.1	B-0.8	A-10.5	AA-<0.1	211	5.2
	TCPO	E-28.5	C-4.9	B-3.9	A-16.5	AA-<0.1	476	5.6
	EPOP	E-12.8	C-0.45	B-0.8	A-3.7	AA-<0.1	146	4.5
	EPIC	E-12.6	C-0.12	B-3.7	A-4.5	AA-<0.1	190	4.0
	OCTO	E- 3.4	C-0.51	B-<0.1	A-5.0	AA-1.8	92	8.3
	CHXO	E- 0.7	C-<0.1	B-<0.1	A-3.1	AA-2.1	52	8.2

Activities expressed as μmol/min/mg of protein (specific activities) or μmol/min (total activity).

Figure 16. Separation of isozymes from liver cytosol of young males by carboxymethylcellulose chromatography. Activities are expressed as nmol/min/μliter of column effluent. Activities with PNBC, DCNB, and CDNB are shown. Additionally, the conductance, which is a function of the increasing concentration of KCl, is shown. The five major peaks represent (from the left) isozymes E plus D, C, B, A, and AA.

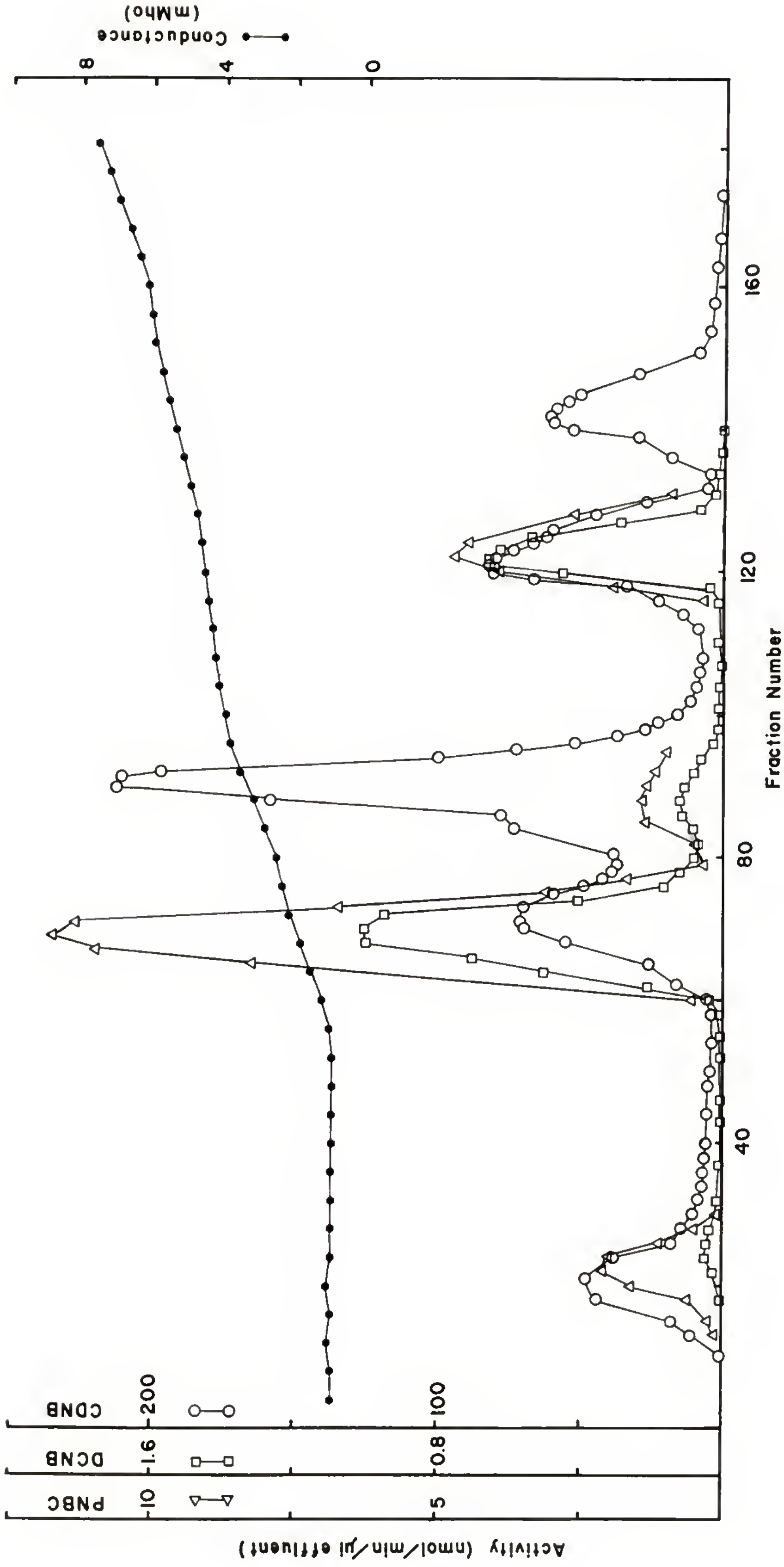
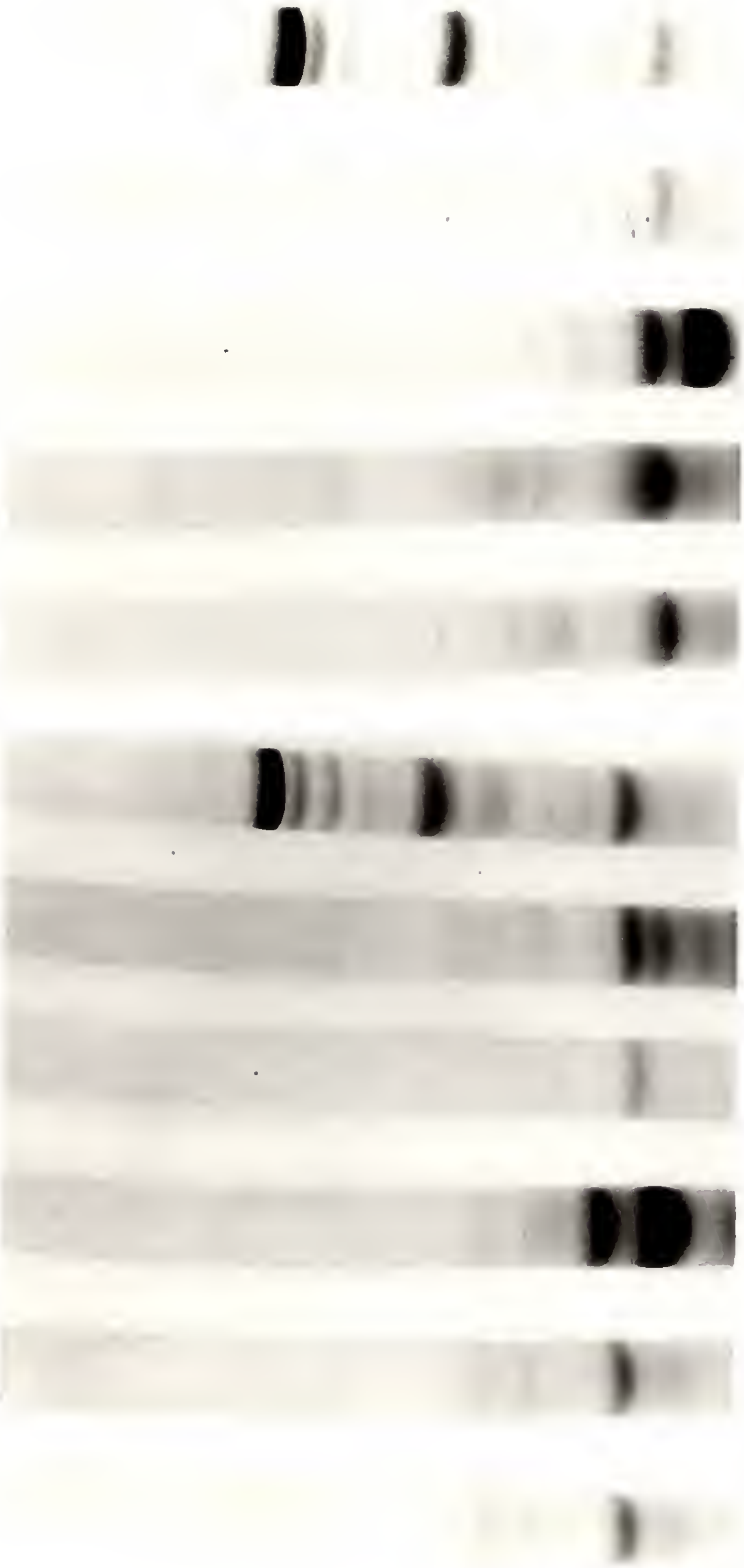

Conductance
(mMho)
8
4
0
Fraction Number
40
80
120
160
Activity (nmol/min/µl effluent)
PNBC
10
5
DCNB
1.6
0.8
CDNB
200
100

Figure 17. SDS-PAGE of purified liver and lung isozymes on 7.5% polyacrylamide gels. *Left panel*, liver isozymes (left to right) E, C, B, A, and AA, and molecular weight standards. *Right panel*, lung isozymes (left to right) E, C, B, and A, and molecular weight standards. Molecular weight standards were trypsingen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000), and bovine serum albumin (66,000).



into its subunits of 23,000-25,000 daltons. Isozymes E, C, and A each formed a single band, implying that both subunits are of identical molecular weight. Isozymes B and AA each formed two bands; those of isozyme AA appeared to be more similar in molecular weight than those of B. In addition, with isozyme B, the smaller subunit (Y_a) appeared to be more predominant than the larger subunit (Y_c); this was not seen with isozyme AA. The subunit composition of the isozymes, and the molecular weights of these subunits, has been previously discussed by Pickett _et_ _al_. (43,44), Beale _et_ _al_. (45), and Mannervik and Jensson (46). These findings about isozyme B support earlier reports of Pickett _et_ _al_. (44, 137, 143) who showed that purified isozyme B formed 23,000 and 25,000 dalton bands with the Y_a band staining more intensely. They concluded that isozyme B (composed of Y_aY_c) and ligandin (composed of Y_aY_a) co-purified. Thus, the purified material has a predominance of Y_a subunits, which explained the more intense staining in the gels. The results of these gels suggested that with isozyme AA the larger subunit predominated.

Specific activities of isozymes. Specific activities of individual isozymes purified from young liver and lung are shown in table 11. For comparison, those data reported by Habig _et_ _al_. (56, 123) are shown; these are essentially identical with the present data. Specific activities with isozymes purified from liver and lung of middle-aged and senescent animals are also shown and closely agree with those from the young tissues. Indeed, the lack of differences indicated that the age-related changes in epoxide metabolism reported in chapter 1 were not due to changes in specific activities of

TABLE 11

SPECIFIC ACTIVITIES OF LIVER AND LUNG ISOZYMES

Organ	Isozyme	Age Group	Substrates								
			STOX	TCPO	EPOP	EPIC	OCTO	CHXO	CDNB	DCNB	PNBC
Liver	E	Young*	nr	nr	nr	nr	nr	nr	0.01	<0.0001	4.1
	C		nr	nr	nr	nr	nr	nr	10	2.0	10.2
	B		nr	nr	nr	nr	nr	nr	11	0.003	0.1
	A		nr	nr	nr	nr	nr	nr	62	4.3	11.4
	AA		nr	nr	nr	nr	nr	nr	14	0.008	0.09
Liver	E	Young	13.5	28.5	12.8	12.6	3.4	0.7	0.04	0.002	4.6
	C		1.1	4.9	0.45	0.1	0.5	<0.1	12.9	1.8	10.9
	B		0.8	3.9	0.8	3.7	<0.1	<0.1	14.0	0.005	0.15
	A		10.5	16.5	3.7	4.5	5.0	3.1	61	3.9	11.2
	AA		<0.1	<0.1	<0.1	<0.1	1.8	2.1	13.2	0.010	0.08
Lung	E	Young	13.5	28.4	12.7	11.5	3.3	0.7	0.009	0.001	4.2
	C		0.9	5.1	0.35	0.1	0.5	<0.1	13.0	1.9	10.2
	B		0.9	4.1	0.6	3.1	0.1	<0.1	13.0	0.003	0.1
	A		10.5	14.5	3.9	4.8	4.8	2.8	59	4.4	11.3
Liver	E	Middle-aged	13.9	27.9	12.9	12.8	3.4	0.8	0.05	0.009	4.5
	C		1.2	4.7	0.4	0.1	0.5	<0.1	14.8	1.5	10.0
	B		0.8	3.7	0.8	3.5	0.1	<0.1	17.5	0.005	0.15
	A		10.7	16.9	3.5	4.5	5.2	3.0	57	3.4	11.1
	AA		<0.1	<0.1	<0.1	<0.1	1.6	2.0	15.3	0.015	0.075

Table 11--extended

Lung	E	Middle-aged	13.6	29.3	12.8	10.9	3.3	0.7	0.01	0.001	4.4
	C		1.0	5.1	0.3	<0.1	0.5	<0.1	10.5	1.6	10.7
	B		1.0	4.1	0.5	3.2	0.1	<0.1	14.0	0.006	0.15
	A		10.5	12.9	3.9	4.9	4.4	2.9	58	3.9	11.4
Liver	E	Senescent	13.1	29.2	12.8	13.3	3.3	0.7	0.03	0	4.2
	C		1.0	5.0	0.3	<0.1	0.5	<0.1	11.9	1.8	10.8
	B		0.9	4.0	0.7	3.0	0.2	<0.1	13.5	0.005	0.09
	A		10.5	16.3	4.0	5.2	4.2	2.5	59	3.7	11.6
	AA		<0.1	<0.1	<0.1	<0.1	1.9	2.0	13.9	0.002	0.07
Lung	E	Senescent	13.0	29.1	12.8	13.5	3.3	0.7	0.02	0.002	3.9
	C		0.9	5.1	0.35	0.1	0.5	<0.1	12.1	2.2	10.8
	B		1.0	3.9	0.5	3.5	0.1	<0.1	11.2	0.003	0.13
	A		10.4	15.9	3.6	5.0	4.9	2.5	53	3.6	11.1

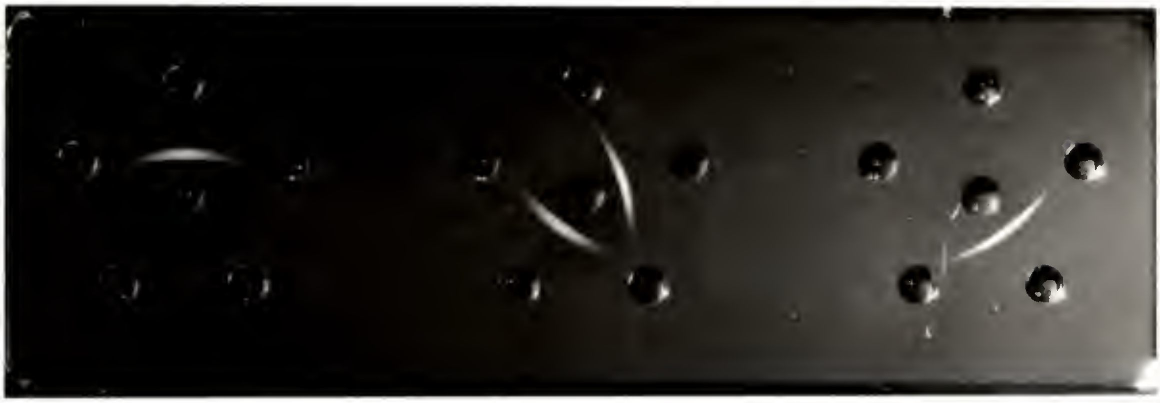
All values are reported in μmol/min/mg of protein and represent means from two experiments run in duplicate. Values with an asterisk were taken from references 56 and 123. Those indicated by "nr" were not reported.

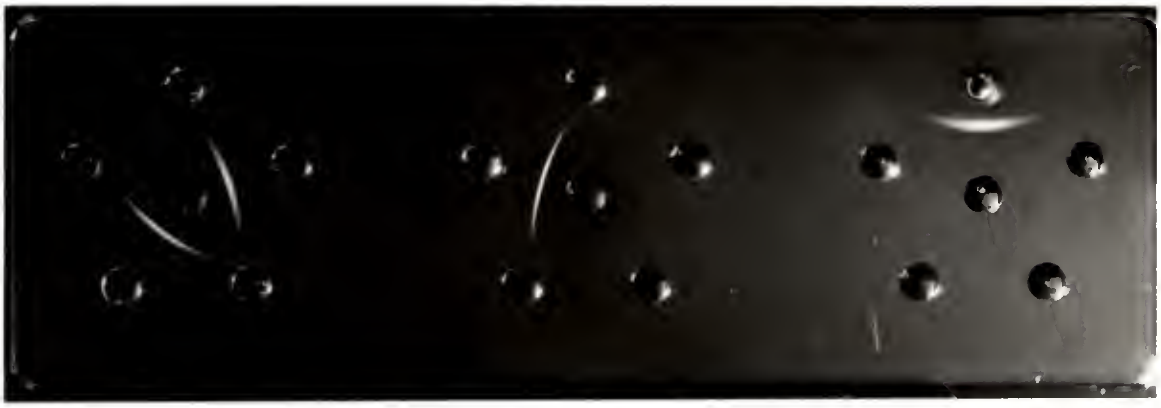
individual isozymes. Instead, another reasonable explanation for the changes in cytosolic metabolism of epoxides or of CDNB, DCNB, PNBC, and EPNP (reported in chapter 2) are changes in the concentrations of the isozymes in the cytosol. The data shown in figures 7-10 and 12-15 are consistent with this hypothesis.

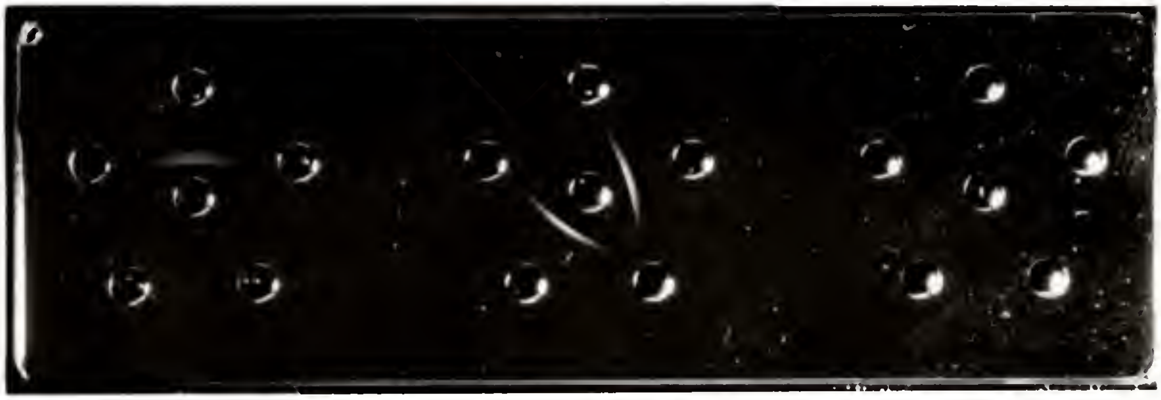
Antisera preparation. Antisera to purified isozymes E, C, B, A, and AA were prepared as described above. Ouchterlony immunodiffusion plates were used to verify the presence of antibodies which cross-reacted with the purified isozymes or with cytosol (figure 18). In addition, immunological cross-reactivity was studied by this method. In figure 19, cross-reactivities of antisera prepared to each liver isozyme with other liver and lung isozymes are shown. Antisera were shown to be cross-reactive with the appropriate purified isozymes and with cytosol. Complete coalescence of precipitin lines in figure 18 indicated that the purified isozymes and those found in the liver cytosol were identical (144). In addition, in the fifth satellite well (10 o'clock) in figure 18, in which 100 mM sodium phosphate (pH 7.4) was placed, no precipitin line was formed, indicating that this was indeed an antigen-antibody reaction. In figure 19, isozymes A and C were shown to be cross-reactive whereas no other isozymes were. These criteria established proper specificity of the antisera. In addition, an isozyme purified from the lung cytosol cross-reacted with (Transferase E)antisera (elicited to isozyme E purified from liver cytosol). This finding, when combined with findings regarding substrate specificities and specific activities (table 11), purification properties, and pI indicated that isozyme E, a major

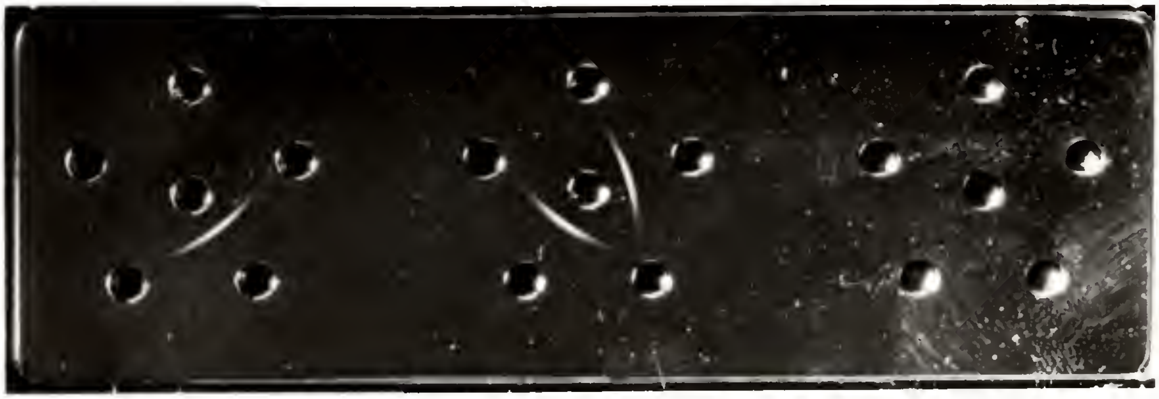
Figure 18. Cross-reactivity of (glutathione S-transferase)antisera with purified isozymes and liver and lung cytosol. Four plates, each with three sets of wells, are shown. The top two plates had liver cytosol (2 o'clock and 7 o'clock) in satellite wells, (left to right in top plate) isozymes B, A, and AA (12 o'clock and 5 o'clock), and (left to right in second plate) isozymes E, C, and E (12 o'clock and 5 o'clock). Center wells in the top plate (left to right) contained antisera to isozymes B, A, and AA. The center wells in the second plate (left to right) contained antisera to isozymes E, C, and E. The center wells of the third plate (left to right) contained antisera to isozymes E and C and those of the bottom plate contained antisera to isozymes B and A; satellite wells contained lung cytosol (2 o'clock and 7 o'clock). Additionally, satellite wells in the third plate contained purified lung isozymes E (left, 12 o'clock and 5 o'clock) and C (center, 12 o'clock and 5 o'clock); those of the bottom plate contained purified lung isozymes B (left, 12 o'clock and 5 o'clock) and A (center, 12 o'clock and 5 o'clock). The right sets of wells in the bottom two plates were unused. In all cases, the well at 10 o'clock contained 100 mM sodium phosphate (pH 7.4). Wells in these Ouchterlony plates held 5 μliter of sample; center-to-center distance between central and satellite wells was 7 mm. The top two plates were developed for 36 hours and refrigerated for 72 hours more, the bottom plates were developed for 24 hours and photographed within 12 hours.

Figure 19. Cross-reactivity of (glutathione S-transferase)antisera with purified isozymes. Four plates, each with three sets of wells, are shown. The top two plates had liver isozymes E (12 o'clock), C (2 o'clock), B (5 o'clock), A (7 o'clock), and AA (10 o'clock) in the satellite wells. Center wells in the top plate (left to right) contained antisera to isozymes E, C, and B. The center wells in the second plate (left to right) contained antisera to isozymes A and AA; that on the right contained isozyme E in the center well with antisera to isozymes E (12 o'clock), C (2 o'clock), B (5 o'clock), A (7 o'clock), and AA (10 o'clock) in satellite wells. The center wells of the third plate contained antisera to isozymes E (left) and C (center) and those of the bottom plate contained antisera to isozymes B (left) and A (center); satellite wells contained lung isozymes E (12 o'clock), C (2 o'clock), B (5 o'clock), and A (7 o'clock). The right sets of wells in the bottom two plates were unused. Wells in these Ouchterlony plates held 5 μliter of sample; center-to-center distance between central and satellite wells was 7 mm. The top two plates were developed for 24 hours and refrigerated for 24 hours, the bottom two plates were developed for 24 hours and photographed within 12 hours.









epoxide-metabolizing form, was present in the lung. Previous work by Guthenberg and Mannervik (53) identified isozymes C, B, and A in the lung and provided evidence for another form which they did not characterize. These data, along with those in chapter 2, provide strong evidence for the existence of isozyme E in the lung.

Immunotitrations of liver and lung cytosol. Immunotitrations of liver and lung cytosol were conducted as described above. In figure 20, immunotitration curves are shown which were generated by adding increasing amounts of (Transferase E)antisera to a constant amount of liver cytosol from each age group. Similarly, immunotitration curves were generated by adding (Transferase E), (Transferase C), (Transferase B), (Transferase A), or (Transferase AA)antisera to liver or lung cytosol. Activity with CDNB, STOX, and TCPO were measured. The immunoprecipitable activities in each age group, relative to the young group, are shown in table 12. These activities are averaged from CDNB, STOX, and TCPO, except with (Transferase E)antisera (STOX and TCPO only) and (Transferase AA)antisera (CDNB only).

Concentrations of isozymes in hepatic and pulmonary cytosol. The ratio of the average rates, for several substrates, of the specific activities with respect to cytosolic protein, to those with respect to purified isozymes, yielded the average fraction of cytosolic protein represented by each glutathione S-transferase isozyme. These concentrations are summarized in table 13. In addition, concentrations per gram of liver and lung are shown in parentheses in table 13. Concentrations of isozymes E and B are averaged from immunotitration and CIEF experiments, those of C and A are from CIEF experiments only,

Figure 20. Immunotitrations of liver cytosol from three age groups. Curves were generated by adding increasing amounts of (Transferase E)antisera to a constant amount of cytosol and measuring resulting activities. Activity is presented as nmol/min/mg of cytosolic protein ± SE. N = 4 for all determinations.

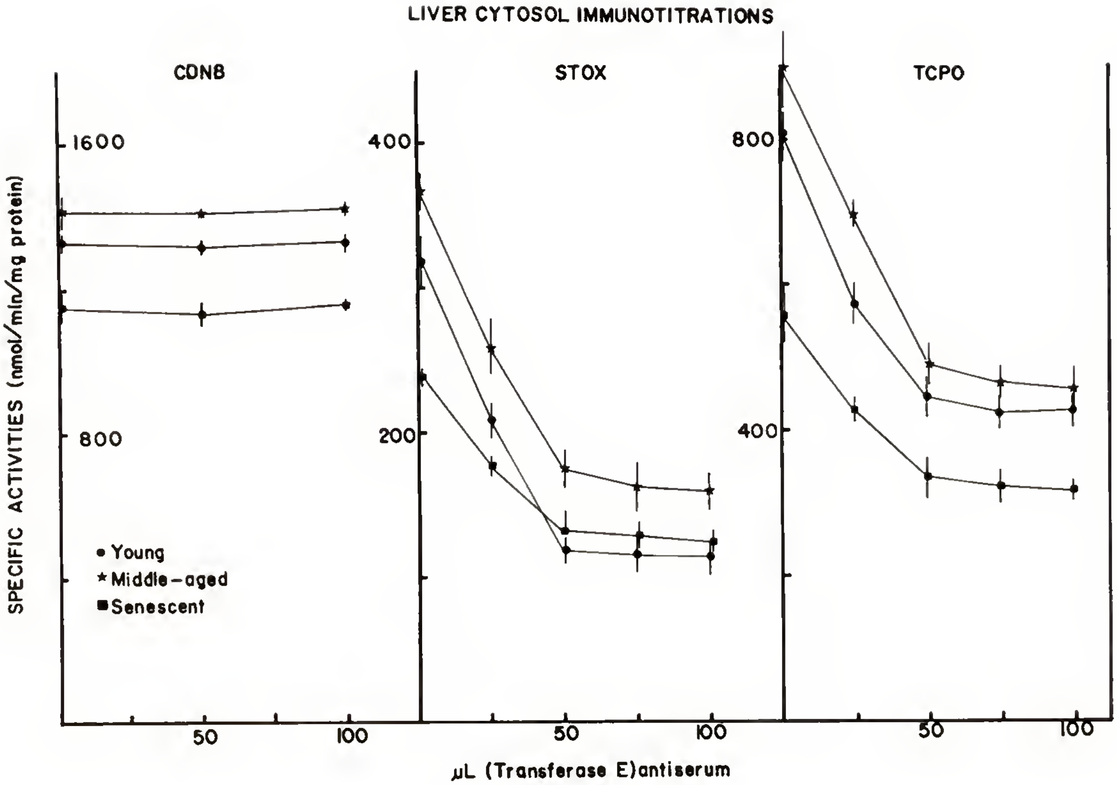

LIVER CYTOSOL IMMUNOTITRATIONS
CDNB
STOX
TCPO
SPECIFIC ACTIVITIES (nmol/min/mg protein)
1600
800
400
200
800
400
• Young
★ Middle-aged
■ Senescent
50
100
50
100
50
100
μL (Transferase E)antiserum

TABLE 12

AGE-RELATED CHANGES IN
IMMUNOPRECIPITABLE HEPATIC AND PULMONARY ISOZYMES

Tissue	Isozyme	3 months old	12 months old	24 months old
Liver	E	1.00	1.17	0.65
	C	1.00	----	----
	B	1.00	1.27	0.58
	A	1.00	----	----
	AA	1.00	1.04	1.15
Lung	E	1.00	0.72	0.66
	C	1.00	----	----
	B	1.00	0.72	0.51
	A	1.00	----	----

Data are averaged from immunotitrations of liver or lung cytosol from each age group. They are expressed relative to the young group; the results with STOX, TCPO, and CDNB were averaged together (except for isozyme E, STOX and TCPO only, and isozyme AA, CDNB only). (Transferase C) and (Transferase A)antisera were cross-reactive, so immunotitration data were not determined for these two isozymes. N = 4 for all determinations.

TABLE 13

CONCENTRATIONS OF ISOZYMES IN HEPATIC AND PULMONARY CYTOSOL

Tissue	Isozyme	3 months old	12 months old	24 months old
Liver	E	0.0112 (0.58)	0.0129 (0.78)	0.0075 (0.44)
	C	0.0209 (1.08)	0.0290 (1.75)	0.0113 (0.66)
	B	0.0549 (2.85)	0.0680 (4.09)	0.0316 (1.85)
	A	0.0068 (0.35)	0.0060 (0.36)	0.0076 (0.44)
	AA	0.0082 (0.42)	0.0085 (0.51)	0.0094 (0.60)
Lung	E	0.00265 (0.061)	0.00208 (0.048)	0.00175 (0.048)
	C	0.00126 (0.029)	0.00097 (0.022)	0.00073 (0.020)
	B	0.00127 (0.029)	0.00089 (0.020)	0.00065 (0.018)
	A	0.00145 (0.033)	0.00145 (0.033)	0.00144 (0.039)

Values are expressed as mg of glutathione S-transferase isozyme per mg of cytosolic protein; those in parentheses are mg of glutathione S-transferase isozyme per gram of tissue.

and those of AA are from immunotitration experiments only. These data support those shown in tables 9 and 12 and figures 7-10 and 12-15. They indicate that during the aging process, concentrations of isozymes E, C, and B increased in the liver of middle-aged rats and then decreased by senescence to below young values. However, isozymes A and AA did not follow this apparent pattern; both appeared to be more concentrated in the senescent liver.

In the lung, isozymes E, C, and B appeared to follow a pattern of decrease from the youth through senescence. In contrast, isozyme A concentrations were unchanged.

Discussion

These results suggest molecular explanations for alterations in the cytosolic metabolism of epoxide substrates (chapter 1) and the more classically used substrates (chapter 2). Several distinct mechanisms could explain those earlier results. For example, during the aging process, the properties of the enzyme molecules themselves could be altered so that they become more or less catalytically efficient with resulting changes in cytosolic metabolism. However, the similarity of specific activities of various purified isozymes indicated that this was not the case. Indeed, other properties such as pI and purification properties were similarly unchanged, indicating that substantial changes in the biochemical or drug-metabolizing properties of the isozymes did not occur during the aging process.

A second reasonable explanation for the age-related changes in cytosolic metabolism of the epoxide and nonepoxide substrates was a change in the concentration or levels of the isozymes. To explore this possibility, antisera were prepared to purified liver isozymes. These antisera were used to precipitate isozymes in the liver and lung cytosol from each age group. Concentrations were derived by the ratio of the specific activities with respect to cytosolic protein to those with respect to purified isozymes. These indicated that the age-related changes in metabolism could be accounted for by changes in the concentrations of the isozymes. One possible weakness in this conclusion is that changes in the immunoreactivity and not in the concentrations of the isozymes may have occured with aging. However, very similar immunotitration curves were generated by using equal amounts of enzyme purified from different age groups (data not shown). An isozyme purified from liver or lung of any age group seemed to have similar immunoprecipitability, specific activities, substrate specificities, and molecular properties; the apparent difference in glutathione conjugation rates between liver and lung and among the three age groups were in isozyme concentration.

In light of recent advances in understanding of the glutathione S-transferases, these results may be further considered. Isozyme subunit composition was defined by Mannervik and Jensson (46) as: isozyme E, unknown; isozyme C, $Y_bY'_b$; isozyme B, Y_aY_c; isozyme A, Y_bY_b; isozyme AA, Y_cY_c. Subunit concentrations per mg of cytosolic protein were analyzed by the following formulas: moles of Y_a = moles of isozyme B, moles of Y_b = 2 (moles of isozyme A) + moles of isozyme C, moles of Y'_b = moles of isozyme C, and moles of Y_c = moles of isozyme B + 2 (moles of isozyme AA). These subunit concentrations were calculated based upon a 48,000 dalton molecular weight for each isozyme. The results are shown in figure 21. These results showed a pattern that is much simpler than that presented when considering changes in isozymes E, C, B, A, and AA in liver and lung and during aging. In the liver or the lung, isozymes Y_a and Y_c and isozymes Y_b and Y'_b followed similar patterns during aging, although clearly patterns differed between the two tissues. In the liver, the Y_a and Y'_b subunits underwent the largest percentage of change (24% and 39% increases from youth to middle age and 43% and 46% decreases from youth to senescence, respectively). In the lung, Y_a and Y_c followed identical patterns of change (no isozyme AA existed in the lung) and Y_a and Y_c showed the largest percentage of change (30% decreases each from youth to middle age and 49% decreases each from youth to senescence). Thus, changes in cytosolic glutathione S-transferase isozyme levels may reflect changes in several subunits with subsequent effects on many transferases. Several strengths and weaknesses exist in this type of interpretation. According to these lines of reasoning, an increase in synthesis of Y'_b subunits might result in more $Y_bY'_b$ (isozyme C) with fewer remaining Y_b subunits subsequently dimerizing to form Y_bY_b (isozyme A). Indeed, by middle age, concentrations of isozyme C were increased in the liver and isozyme A slightly decreased. By senescence, a decrease in Y'_b subunit production would cause a reversal of this pattern: less $Y_bY'_b$ with more subsequent Y_bY_b dimerization, or less isozyme C and more isozyme A (as was found). Similarly, an increase in synthesis of Y_a subunits might result in more Y_aY_c (isozyme B) with less Y_c remaining for subsequent dimerization to Y_cY_c (isozyme AA). In the liver, an increase in isozyme B was found at middle age, but no corresponding decrease in

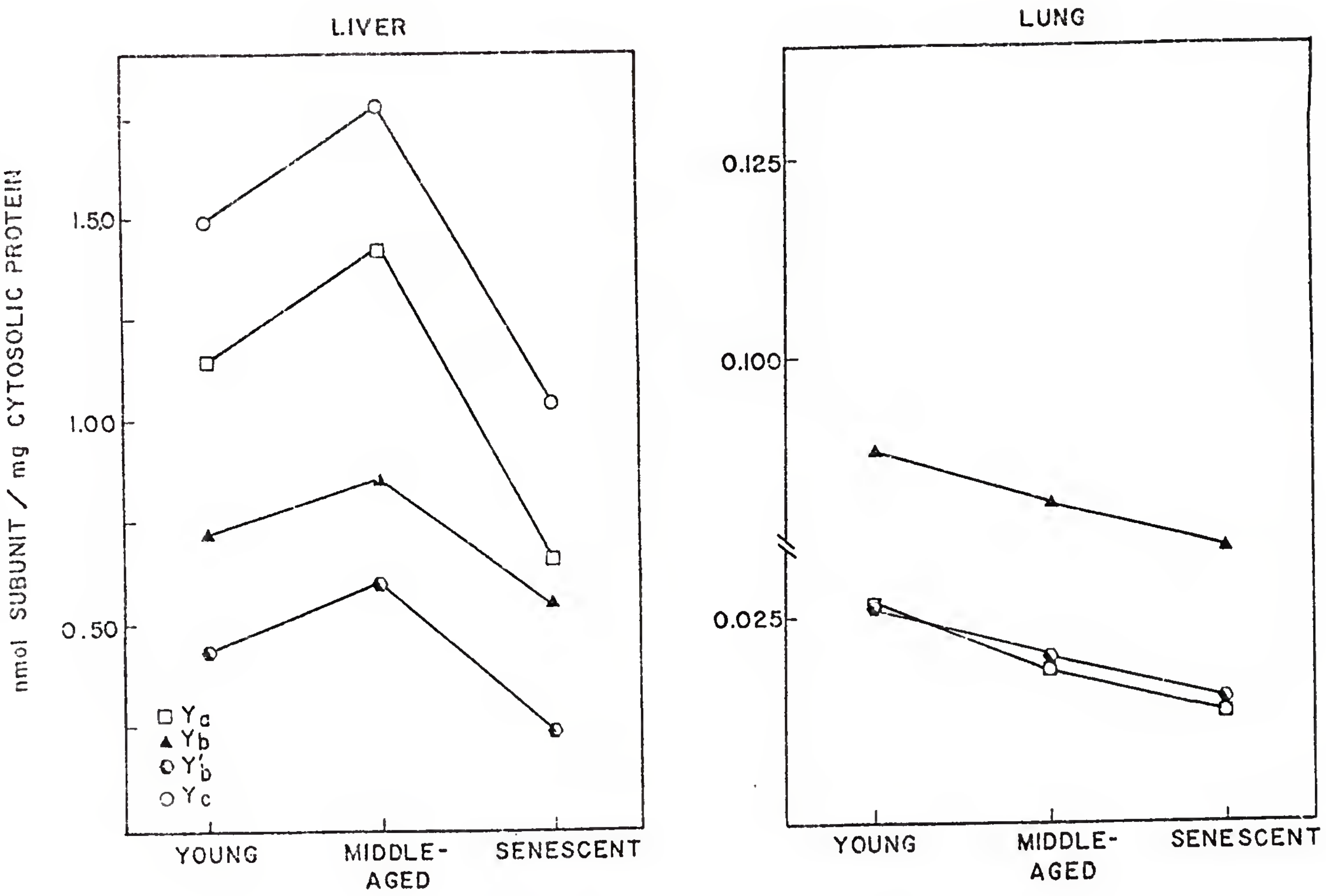


Figure 21. Changes in subumit composition of liver and lung cytosol with age.

isozyme AA was apparent. By senescence, a decrease in Y_a would result in less Y_aY_c and more Y_cY_c, or less isozyme B and more isozyme AA, as occurred in the liver. As the subunit composition of isozyme E is currently unknown, analysis of its subunit changes and of the contribution to this scheme during the aging process is not possible. This interpretation, that subunits, and not the isozymes themselves, were modulated during the aging process is quite tempting to consider.

However attractive these proposed changes in subunits may seem, they may be at best oversimplified, or perhaps inaccurate. For example, the increases in isozyme C in the liver in middle age are not stoichiometrically matched by decreases in isozyme A, and vice versa in the senescent group, even when the relative concentrations are considered. The same imbalances apply when isozymes B and AA in the liver and A and C in the lung are considered. It is possible, as suggested by Pickett et al. (143) that in addition to a subunit such as Y_a competing with Y_c for formation of Y_aY_c or Y_cY_c, Y_a may also dimerize to form Y_aY_a (which they defined as ligandin, a form of isozyme B). Thus, competing homodimerization reactions with these subunits may affect the potential for heterodimerization. In addition, the scheme of subunit composition proposed by Mannervik and Jensson (46) may be oversimplified. For example, if isozymes B and AA share a common Y_c subunit, and AA is a homodimer of Y_cY_c (to which antibodies were raised), then immunological cross-reactivity between isozymes B and AA might be expected. However, neither this work nor that reported earlier (56,57) indicated that these isozymes were cross-reactive.

Also, the supposition that isozyme AA is a Y_cY_c homodimer is not supported by the SDS-PAGE results, which show that two bands of close, but not identical, molecular weight constitute it.

Nevertheless, the data support the suggestion that during the aging process, changes in the concentrations of the isozymes occurred which altered hepatic and pulmonary GSH conjugation. One reasonable explanation for these changes in isozyme concentration was changes in subunit concentrations. In the liver, levels of isozymes E, C, and B were increased in the middle-aged group and decreased in the senescent group, whereas isozymes A and AA were unchanged (or perhaps slightly decreased) in middle age and increased by senescence. In the lung, E, C, and B followed a pattern of consistent decrease from youth through senescence, whereas isozyme A concentrations were unchanged. These data also indicated that isozyme E, a major epoxide-metabolizing form in the liver, was present in the lung. No previous work had shown this, although evidence for an unidentified form in the lung has been presented (39).

Thus, these changes in isozyme concentrations during the aging process affect electrophile metabolism, as reported in chapters 1 and 2. Inasmuch as the organism is exposed to many electrophilic chemicals, and inasmuch as there are many other functions of the glutathione S-transferases, these changes during the aging process may reasonably alter the animal's survival ability.

CONCLUSIONS

The results presented in this dissertation demonstrate that age-related alterations in electrophile metabolism by the cytosolic glutathione S-transferases of rat liver and lung cytosol occurred. These results showed tissue-, sex-, and substrate-specific alterations. By senescence, specific activities measured with all epoxide substrates tested were decreased in liver cytosol from males. With lung cytosol from males and liver and lung from females, specific activities with only some substrates were changed by the aging process. In addition, changes in the concentration of protein and in the weights of liver and lung during aging altered the total epoxide-metabolizing capacities. Finally, the affinity of the cytosolic glutathione S-transferases for the epoxide styrene oxide were altered by senescence in liver and lung cytosol from males, but not females. These results indicated that selective changes in epoxide metabolism occurred, and that the quantities of these changes depended on the epoxide substrate, the tissue, and the sex of the animal. They also indicated that alterations in epoxide toxicity with increasing age might depend on the properties of the epoxide and its dose or concentration, the tissue which is exposed, and the sex of the animal.

Inasmuch as the specific activities of individual glutathione S-transferase isozymes toward CDNB, DCNB, PNBC, and EPNP are known, they were used to further examine age-related changes in electrophile metabolism to provide indications of age-related changes in levels or activities of individual isozymes. Similarly to the results found with the epoxide substrates, the age-related alterations in the metabolism of CDNB, DCNB, PNBC, and EPNP were tissue- and sex-specific. Changes were most commonly found with EPNP, DCNB, and PNBC, which are each metabolized by a few isozymes. With CDNB, specific activities tended not to be changed with increasing age except with liver cytosol from females, perhaps because most of the isozymes contribute to its biotransformation. Additionally, age-related alterations occurred in the affinity of liver cytosol from males, but not lung cytosol from males or liver and lung cytosol from females, for CDNB.

The mechanism for these age-related alterations in electrophile metabolism appeared to be changes in the concentrations of various isozymes. With glutathione S-transferase isozymes purified from liver and lung cytosol of males, no changes were found in the specific activities measured with epoxide or nonepoxide substrates. Thus, the possibility that changes in the catalytic properties of individual isozymes caused changes in electrophile metabolism seemed improbable. Instead, column isoelectric focusing and immunotitrations of cytosol indicated that isozyme concentrations changed with increasing age. In liver cytosol from males, concentrations of isozymes E, C, and B increased through middle-age and then decreased by senescence (relative to the young group). By senescence, concentrations of isozymes A and AA were increased above those measured in the young group. In lung cytosol from males, concentrations of isozymes E, C and B followed a pattern of decrease from youth through senescence, whereas isozyme A levels were unchanged. This dissertation reports the first identification of isozyme E in the lung.

Although subunit composition of individual isozymes has not been conclusively established, analysis of the above data on the basis of age-related changes in subunits indicated that changes in the levels of the subunits may selectively alter the concentrations of individual isozymes. The Y_a, Y_b, Y'_b, and Y_c subunits in the liver were all increased in middle-age and decreased in senescence (relative to the young group). In the lung, all followed a pattern of decrease from youth through senescence. In addition, relative subunit levels differed between liver and lung; Y_a and Y_c were predominant in the liver whereas Y_b predominated in the lung. The contribution of isozyme E subunits to this scheme was not evaluated because subunit composition of isozyme E has not been studied.

These results demonstrate that during the aging process, concentrations of most of the glutathione S-transferase isozymes were altered in the liver and lung cytosol of male Fischer 344 rats. In addition, changes in the metabolism of epoxide and nonepoxide substrates indicated that changes in isozyme concentrations in liver and lung cytosol of female F344 rats also occurred during aging. These glutathione S-transferase isozymes function in many capacities, including selenium-independent glutathione peroxidases, intracellular binding proteins, steroid isomerases, in the synthesis of leukotriene B_4, and as glutathione S-transferases. Thus, age-related alterations in their levels or concentrations may affect several biological systems. In addition, age-related alterations in biotransformation systems which affect glutathione S-transferase substrates, such as epoxide hydrolase or the cytochromes P-450, may also change with increasing age. Finally, changes in other cellular systems which prevent or repair damage to important cellular macromolecules, such as DNA or proteins, are important when considering the relationship of changes in the concentrations of the glutathione S-transferases to the toxicity or carcinogenicity of xenobiotics.

The field of aging research is one of complex interactions of multiple systems. The results presented in this dissertation express changes in the concentrations and resulting activities of the cytosolic glutathione S-transferases in liver and lung cytosol during aging. If the process of xenobiotic toxicity or carcinogenesis involves a threshhold which must be exceeded before deleterious effects are demonstrated, then these changes may assume importance. In addition, these changes in the cytosolic glutathione S-transferases, and those in other biotransformation systems, will need to be simultaneously evaluated as further information is gained. As a result, the effect of aging on drug metabolism and toxicity may be more fully explained and predicted.

REFERENCES

1. Schmucker, D.L. and Wang, R.K., Experimental Gerontology: 15, 321-329, 1980.

2. Birnbaum, L.S. and Baird, M.B., Experimental Gerontology: 13, 299-303, 1978.

3. Birnbaum, L.S. and Baird, M.B., Experimental Gerontology: 13, 469-477, 1978.

4. Kao, J. and Hudson, P., Biochemical Pharmacology: 29, 1191-1194, 1980)

5. Rikans, L.E. and Notley, B.A., Journal of Pharmacology and Experimental Therapeutics: 220, 574-578, 1982.

6. Ariyoshi, T., Kazumitsu, T. and Hamasaki, K., Journal of Pharmacobio-Dynamics: 4, 664-669, 1981.

7. Birnbaum, L.S. and Baird, M.B., Chemico-Biological Interactions: 26, 245-256, 1979.

8. Baird, M.B., Zimmerman, J.A., Massie, H.R. and Pacilo, L.V. Experimental Gerontology: 11, 161-165, 1978.

9. Knook, D.L., Proceedings of the Society for Experimental Biology and Medicine: 165, 170-177, 1980.

10. Stohs, S.J., Al-Turk, W.A. and Angle, C.R., Biochemical Pharmacology: 31, 2113-2116, 1982.

11. Wilson, P.D., Watson, R. and Knook, D.L., Gerontology: 28, 32-43, 1982.

12. Chiang, J.Y.L., DiLella, A.G. and Steggles, A.W., Molecular Pharmacology: 23, 244-251, 1983.

13. Wood, A.J.J., Vestal, R.E., Wilkinson, G.R., Branch, R.A. and Shand, D.G., Clinical Pharmacology and Therapeutics: 26, 16-20, 1979.

14. Greenblatt, D.J., Divoll, M., Abernathy, D.R., Harmatz, J.S. and Shader, R.I., Journal of Pharmacology and Experimental Therapeutics: 220, 120-126, 1982.

15. Vestal, R.E., Wood, A.J.J., Branch, R.A., Shand, D.G. and Wilkinson, G.R., Clinical Pharmacology and Therapeutics: 26, 8-15, 1979.

16. Jusko, W.J., Gardner, M.J., Mangione, A., Schentas, J.J., Koup, J.R. and Vance, J.W., Journal of Pharmaceutical Science: 68, 1358-1366, 1979.

17. Greenblatt, D.J., Allen, M.D., Locniskar, A., Harmatz, J.S. and Shader, R.I., Clinical Pharmacology and Therapeutics: 26, 103-113, 1979.

18. Kangas, L., Iisalo, E., Kanto, J., Lehtinen, V., Pynnonen, S., Ruikka, I., Salminen, J., Sillanpaa, M. and Syvalahti, E., European Journal of Clinical Pharmacology: 15, 163-170, 1979.

19. Farah, F., Taylor, W., Rawlins, M.D. and James, O., British Medical Journal: 2, 155-156, 1977.

20. Thacker, D.R., Yagi, H., Lu, A.Y.H., Levin, W., Conney, A. and Jerina, D.M., Proceedings of the National Acacemy of Sciences, USA: 73, 3381-3385, 1976.

21. van Bladeren, P.J., Breimer, D.D., Rotteveal-Smijs, G.M.T., deJong, R.A.W., Buijis, W., van der Gen, A. and Mohn, G.R., Biochemical Pharmacology: 29, 2975-2982, 1980.

22. Baird, M.B. and Birnbaum, L.S., Cancer Research: 39, 4752-4755, 1979.

23. Jayaraj, A. and Richardson, A., Mechanisms of Ageing and Development: 17, 163-171, 1981.

24. Morgenstern, R., Guthenberg, C., Mannervik, B., DePierre, J.W. and Ernster, L., Cancer Research: 42, 4215-4221, 1982.

25. Hesse, S., Jernstrom, B., Martinez, M., Moldeus, P., Christodoulides, L. and Ketterer, B., Carcinogenesis: 3, 757-760, 1982.

26. Lieberman, M.W. in Environmental Pathology: An Evolving Field, eds. Hill, R.B. and Terzian, J.A., pp. 53-76, Alan R. Liss, Inc., New York, 1982.

27. Niedermuller, H., Mechanisms of Ageing and Development: 19, 259-271, 1982.

28. Bowman, P.D., Meek, R.L. and Daniel, C.W., Mechanisms of Ageing and Development: 5, 251-257, 1976.

29. Icard, C., Beaupain, R., Diatloff, C. and Macieira-Coelho, A., Mechanisms of Ageing and Development: 11, 269-278, 1979.

30. Lambert, B., Ringborg, U. and Skoog, L., Cancer Research: 39, 2792-2795, 1979.

31. Jakoby, W.B. and Habig, W.H. in Enzymatic Basis of Detoxification, ed., Jakoby, W.B., pp. 63-95, Academic Press, New York, 1980.

32. Kamisaka, K., Habig, W.H., Ketley, J.N., Arias, I.M. and Jakoby, W.B., European Journal of Biochemistry: 60, 153-161, 1975.

33. Awasthi, Y.C., Dao, D.D. and Saneto, R.P., Biochemical Journal: 191, 1-10, 1980.

34. Warholm, M., Guthenberg, C., Mannervik, B. and vonBahn, C., Biochemical and Biophysical Research Communications: 98, 512-519, 1981.

35. Marcus, C.J., Habig, W.H. and Jakoby, W.B., Archives of Biochemistry and Biophysics: 188, 287-293, 1978.

36. Guthenberg, C. and Mannervik, B., Biochimica et Biophysica Acta: 661, 255-260, 1981.

37. Prohaska, J.R., Biochimica et Biophysica Acta: 611, 87-98, 1980.

38. Irwin, C., O'Brien, J.K., Chu, P., Townsend-Parchman, J.K., O'Hara, P. and Hunter, F.E., Archives of Biochemistry and Biophysics: 205, 122-131, 1980.

39. Benson, A.M. and Talalay, P., Biochemical and Biophysical Research Communications: 69, 1073-1079, 1976.

40. Benson, A.M., Talalay, P., Keen, J.H. and Jakoby, W.B., Proceedings of the National Academy of Sciences, USA: 74, 158-162, 1977.

41. Jakoby, W.B., Advances in Enzymology: 46, 383-414, 1978.

42. Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M. and Jakoby, W.B., Proceedings of the National Academy of Sciences, USA: 71, 3879-3882, 1974.

43. Pickett, C.B., Wells, W., Lu, A.Y.H. and Hales, B.F., Biochemical and Biophysical Research Communications: 99, 1002-1010, 1981.

44. Pickett, C.B., Donohue, A.M., Lu, A.Y.H. and Hales, B.F., Archives of Biochemistry and Biophysics: 215, 539-543, 1982.

45. Beale, D., Ketterer, B., Carne, T., Meyer, D. and Taylor, J.B., European Journal of Biochemistry: 126, 459-463, 1982.

46. Mannervik, B. and Jensson, H., Journal of Biological Chemistry: 257, 9909-9912, 1982.

47. Bhargava, M.M., Ohmi, N., Listowsky, I. and Arias, I.M., Journal of Biological Chemistry: 255, 718-723, 1980.

48. Strange, R.C., Cramb, R., Hayes, J.D. and Percy-Robb, I.W., Biochemical Journal: 165, 425-429, 1977.

49. Bass, N.M., Kirsch, R.E., Tuff, S.A., Marks, I. and Saunders, S.J., Biochimica et Biophysica Acta: 492, 163-175, 1977.

50. Dierickx, P.J. and Yde, M.V., Research Communications in Chemical Pathology and Pharmacology: 37, 385-394, 1982.

51. Pabst, M.J., Habig, W.H. and Jakoby, W.B., Journal of Biological Chemistry: 249, 7140-7150, 1974.

52. Bhargava, M.M., Listowsky, I. and Arias, I.M., Journal of Biological Chemistry: 253, 4112-4115, 1978.

53. Guthenberg, C. and Mannervik, B., Biochemical and Biophysical Research Communications: 86, 1304-1310, 1979.

54. Hales, B.F., Jaeger, V. and Neims, A.H., Biochemical Journal: 175, 937-943, 1978.

55. Kaplowitz, N., Clifton, G., Kuhlenkamp, J. and Wallin, J.D., Biochemical Journal: 148, 243-248, 1976.

56. Habig, W.H., Pabst, M.J. and Jakoby, W.B., Archives of Biochemistry and Biophysics: 175, 710-716, 1976.

57. Hayes, J.D. and Clarkson, G.H.D., Biochemical Journal: 207, 459-470, 1982.

58. Kalinyak, J.E. and Taylor, J.M., Journal of Biological Chemistry: 257, 523-530, 1982.

59. Keen, J.H., Habig, W.H. and Jakoby, W.B., Journal of Biological Chemistry: 251, 6183-6188, 1976.

60. Gould, E.S. in Mechanisms and Structure in Organic Chemistry, ed. Gould, E.S., Holt, Rhinehart and Winston, New York, pp. 212-239, 1958.

61. Pirie, N.W. and Pinhey, K.G., Journal of Biological Chemistry: 84, 321-323, 1929.

62. Keen, J.H. and Jakoby, W.B., Journal of Biological Chemistry: 253, 5654-5657, 1978.

63. Jakoby, W.B., Ketley, J.N. and Habig, W.H. in Glutathione: Metabolism and Function, eds. Arias, I.M. and Jakoby, W.B., Raven Press, New York, pp. 213-223, 1976.

64. Jakoby, W.B., Habig, W.H., Keen, J.H., Ketley, J.N. and Pabst, M.J. in Glutathione: Metabolism and Function, pp. 189-211, eds. Arias, I.M. and Jakoby, W.B., Raven Press, New York, 1976.

65. Hayakawa, T., Udenfriend, S., Yagi, H. and Jerina, D.M., Archives of Biochemistry and Biophysics: 170, 438-451, 1975.

66. Ryan, A.J., James, M.O., Ben-Zvi, Z., Law, F.C.P. and Bend, J.R., Environmental Health Perspectives: 17, 135-144, 1976.

67. Ryan, A.J. and Bend, J.R., Drug Metabolism and Disposition: 5, 363-367, 1977.

68. Pacifici, G.M. and Rane, A., Drug Metabolism and Disposition: 10, 302-305, 1982.

69. James, M.O., Fouts, J.R. and Bend, J.R., Biochemical Pharmacology: 25, 187-193, 1976.

70. James, M.O., Foureman, G.L., Law, F.C. and Bend, J.R., Drug Metabolism and Disposition: 5, 19-28, 1977.

71. Steele, J.W., Yagen, B., Hernandez, O., Cox, R.H., Smith, B.R. and Bend, J.R., Journal of Pharmacology and Experimental Therapeutics: 219, 35-41, 1981.

72. Yagen, B., Hernandez, O. and Bend, J.R., Chemico-Biological Interactions: 34, 57-67, 1981.

73. Delbressine, L.P.C., van Bladeren, P.J., Smeets, F.L.M. and Seuter-Berlage, F., Xenobiotica: 11, 589-594, 1981.

74. Milvy, P. and Garro, A.J., Mutation Research: 40, 15-18, 1976.

75. Loprieno, N., Abbondandolo, A., Barale, R., Baroncelli, S., Bonatti, S., Bronzetti, G., Cammellini, A., Crosi, C., Corti, G., Frezza, D., Leporini, C., Mazzaccaro, A., Nieri, R., Rosellini, D. and Rossi, A.M., Mutation Research: 40, 317-324, 1976.

76. Dowty, B.J., Carlisle, D.R. and Laseter, J.L., Environmental Science and Technology: 9, 762-766, 1975.

77. Leibman, K.C. and Ortiz, E., Journal of Pharmacology and Experimental Therapeutics: 173, 242-246, 1970.

78. Oesch, F. and Daly, J., Biochemical and Biophysical Research Communications: 46, 1713-1727, 1972.

79. Fakhouri, G. and Jones, A.R., Australian Journal of Pharmaceutical Science: 8, 11-14, 1979.

80. Weigel, W.W., Plotnick, H.B. and Conner, W.L., Research Communications in Chemical Pathology and Pharmacology: 20, 275-286, 1978.

81. Eder, E., Nendecker, T., Lutz, D and Henschler, D., Biochemical Pharmacology: 29, 993-998, 1980.

82. Knapp, A.G.A.C., Voogd, C.E. and Kramers, P.G.N., Mutation Research: 101, 198-208, 1982.

83. Marks, T.A., Gerling, F.S. and Staples, R.E., Journal of Toxicology and Environmental Health: 9, 87-96, 1982.

84. Lawrence, W.H., Malik, M., Turner, J.E. and Autian, J., Journal of Pharmaceutical Science: 61, 1712-1717, 1972.

85. Hemminki, K., Paasivirta, J., Kurkirinne, T. and Virkki, L., Chemico-Biological Interactions: 30, 259-270, 1980.

86. Greene, E.J., Friedman, M.A., Sherrod, J.A. and Salerno, A., Mutation Research: 67, 9-19, 1979.

87. Friedman, M.A., Greene, E.J., Sherrod, J.A. and Salerno, A.J., Pharmacologist: 20, 154-159, 1978.

88. Terrill, J.B., Lee, K.P., Culik, R. and Kennedy, G.L., Toxicology and Applied Pharmacology: 64, 204-212, 1982.

89. Maynert, E.W., Foreman, R.L. and Watabe, T., Journal of Biological Chemistry: 245, 5234-5238, 1970.

90. van Bladeren, P.J., Breimer, D.D., Seghers, C.J.R., Vermeulen, N.P.E., van der Gen, A. and Cauvet, J., Drug Metabolism and Disposition: 9, 207-211, 1981.

91. Oesch, F., Kaubisch, N., Jerina, D.M. and Daly, J.W., Biochemistry: 26, 4858-4866, 1971.

92. VanDuuren, B.L., Journal of Environmental Pathology and Toxicology: 3, 11-34, 1980.

93. Arias, I.M., Fleischner, G., Kirsch, R., Mishkin, S. and Gatmaitan, Z., in Glutathione: Metabolism and Function, pp. 175-188, eds. Arias, I.M. and Jakoby, W.B., Raven Press, New York, 1976.

94. Hales, B.F. and Neims, A.H., Biochemical Journal: 160, 231-236, 1976.

95. Litwack, G., in Glutathione: Metabolism and Function, eds. Arias, I.M. and Jakoby, W.B., Raven Press, New York, pp. 285-299, 1976.

96. Reyes, H., Levi, A.J., Gatmaitan, Z. and Arias, I.M., Journal of Clinical Investigation: 50, 2242-2245, 1971.

97. Hales, B.F and Neims, A.H., Biochemical Journal: 160, 223-229, 1976.

98. Hales, B.F., Jain, R. and Robaire, B., Biochemical Pharmacology: 31, 2389-2393, 1982.

99. Bethea, C.L. and Walker, R.F., Journal of Gerontology: 34, 21-27, 1979.

100. Jerina, D.M., Daly, J.W., Witkop, B., Zaitzman-Nirenberg, P. and Udenfriend, S., Biochemistry: 9, 147-155, 1970.

101. Huberman, E., Sachs, L., Yuang, S.K., and Gelboin, H.V., Proceedings of the National Academy of Sciences, USA: 73, 607-611, 1976.

102. Kapitulnik, J., Wislocki, P.G., Levin, W., Yagi, H., Jerina, D.M., and Conney, A.H., Cancer Research: 38, 354-358, 1978.

103. Hesse, S., Jernstrom, B., Martinez, M., Guenther, T. and Orrenius, S., Biochemical and Biophysical Research Communications: 94, 612-617, 1980.

104. Jernstrom, B., Babson, J.R., Moldeus, P., Holmgren, A. and Reed, D.J., Carcinogenesis: 3, 861-866, 1982.

105. Storck, W.J., Chemical Engineering News: 56, 32-36, 1978.

106. VanDuuren, B.L., Nelson, N., Orris, L., Palmes, E.D. and Schmitt, F.L., Journal of the National Cancer Institute: 31, 41-55, 1963.

107. Fries, J.F., New England Journal of Medicine: 303, 130-135, 1980.

108. Rowe, J.W., New England Journal of Medicine: 297, 1332-1336, 1977.

109. Koch-Weser, J., New England Journal of Medicine: 306, 1081-1088, 1982.

110. Coleman, G.L., Barthold, S.W., Osbaldiston, G.W., Foster, S.J. and Jonas, A.M., Journal of Gerontology: 32, 258-278, 1977.

111. Masoro, E.J., Experimental Aging Research: 6, 219-233, 1980.

112. Hayakawa, T., Lemahieu, R.A. and Udenfriend, S., Archives of Biochemistry and Biophysics: 162, 223-230, 1974.

113. Pachecka, J., Gariboldi, P., Cantoni, L., Belvedere, G., Mussini, E. and Salmona, M., Chemico-Biological Interactions: 27, 313-321, 1979.

114. Watabe, T., Hiratsuka, A., Ozawa, N. and Isobe, M., Biochemical Pharmacology: 30, 390-392, 1981.

115. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Journal of Biological Chemistry: 193, 265-275, 1951.

116. Engelman, G.L., Richardson, A., Katz, A. and Fierer, J.A., Mechanisms of Ageing and Development: 16, 385-395, 1981.

117. Kritchevsky, D., Proceedings of the Society of Experimental Biology and Medicine: 165, 193-199, 1980.

118. Van Bezooijen, C.F.A., Grell, T. and Knook, D.L., Mechanisms of Ageing and Development: 6, 293-304, 1977.

119. Viskup, R.W., Baker, M., Holbrook, J.P. and Penniall, R., Experimental Aging Research: 5, 487-496, 1979.

120. Cook, J.R. and Beutwo, D.E., Mechanisms of Ageing and Development: 17, 41-52, 1981.

121. Fjellstedt, T.A., Allen, R.H., Duncan, B.K. and Jakoby, W.B., Journal of Biological Chemistry: 248, 3702-3707, 1973.

122. Patel, J.M., Wood, J.C. and Leibman, K.C., Drug Metabolism and Disposition: 8, 305-308, 1980.

123. Habig, W.H., Pabst, M.J. and Jakoby, W.B., Journal of Biological Chemistry: 249, 7130-7139, 1974.

124. Dahlen, S.-E., Bjork, J., Hedqvist, P., Arfors, K.-E., Hammarstrom, S., Lindgren, J.-A. and Samuellson, B., Proceedings of the National Academy of Sciences, USA: 78, 3887-3891, 1981.

125. Fleischner, G., Robbins, J. and Arias, I.M., Journal of Clinical Investigation: 51, 677-684, 1972.

126. James, S.P. and Pheasant, A.E., Xenobiotica: 8, 207-217, 1978.

127. Stohs, S.J., Al-Turk, W.A., Angle, C.R. and Heinicke, R.J., General Pharmacology: 13, 519-522, 1982.

128. Kitahara, A., Ebina, T., Ishikawa, T., Soma, Y., Sata, K. and Kanai, S. in Liver and Aging -- 1982, Liver and Drugs, ed. Kitani, K., pp. 135-143, Elsevier Biomedical Press, Amsterdam, Netherlands, 1982.

129. Habig, W.H., Keen, J.H. and Jakoby, W.B., Biochemical and Biophysical Research Communications: 64, 501-506, 1975.

130. Mukhtar, H., Lee, I.P., Foureman, G.L. and Bend, J.R., Chemico-Biological Interactions: 22, 153-165, 1978.

131. Hales, B.F. and Neims, A.H., Biochemical Pharmacology: 26, 555-556, 1977.

132. Mukhtar, H. and Bresnick, E., Biochemical Pharmacology: 25:1081-1084, 1976.

133. Clifton, G. and Kaplowitz, N., Biochemical Pharmacology: 27, 1284-1287, 1978.

134. Guthenberg, C., Morgenstern, R., DePierre, J.W. and Mannervik, B., Biochimica et Biophysica Acta: 631, 1-10, 1980.

135. Kaplowitz, N., Kuhlenkamp, J. and Clifton, G., Biochemical Journal: 146, 351-356, 1975.

136. Robaire, B. and Hales, B.F., Biology of Reproduction: 26, 559-565, 1982.

137. Pickett, C.B., Telakowski-Hopkins, C.A., Donohue, A.M., Lu, A.Y.H. and Hales, B.F., Biochemical and Biophysical Research Communications: 104, 611-619, 1982.

138. Sparnins, V.L., Venegas, P.O. and Wattenberg, L.W., Journal of the National Cancer Institute: 68, 493-495, 1982.

139. Sparnins, V.L., Chuan, J. and Wattenberg, L.W., Cancer Research: 42, 1205-1207, 1982.

140. Cobb, D., Boehlert, C., Lewis, D. and Armstrong, R.N., Biochemistry: 22, 805-812, 1983.

141. Stohs, S.J., Al-Turk, W.A. and Angle, C.R., Toxicologist: 1, 143, 1981.

142. Weber, K. and Osborn, M., Journal of Biological Chemistry: 244, 4406-4412, 1969.

143. Pickett, C.B., Telakowski-Hopkins, C.A., Donohue, A.M. and Lu, A.Y.H., Archives of Biochemistry and Biophysics: 221, 89-98, 1983.

144. Munoz, J. in Methods in Immunology and Immunochemistry, Volume III, eds. Williams, C.A. and Chase, M.W., pp. 146-168, Academic Press, New York, 1971.

SUPPLEMENTARY REFERENCES

Leibman, K.C. and Spearman, M.E., Federation Proceedings: 42, 1142, 1983.

Spearman, M.E. and Leibman, K.C., Federation Proceedings: 42, 1293, 1983.

Spearman, M.E. and Leibman, K.C., Pharmacologist, in press, 1983.

Spearman, M.E. and Leibman, K.C., "Hepatic and Pulmonary Cytosolic Metabolism of Epoxides: Effects of Aging on Conjugation with Glutathione", manuscript in preparation for submission to Life Sciences, 1983.

Spearman, M.E. and Leibman, K.C., "The Effects of Aging on Hepatic and Pulmonary Glutathione S-Transferase Activities in Male and Female Fischer 344 Rats", manuscript in preparation for submission to Biochemical Pharmacology, 1983.

Spearman, M.E. and Leibman, K.C., "Aging Selectively Alters Glutathione S-Transferase Isozymes in Liver and Lung Cytosol", manuscript in preparation for submission to Drug Metabolism and Disposition, 1983.

BIOGRAPHICAL SKETCH

Marshall Edward Spearman was born on November 8, 1953, in the sleepy little town of Mount Vernon, Ohio. After a dozen years of traditional midwestern life, his family moved to Columbia, South Carolina, where they resided about one-third of a decade. He then completed his secondary school education in Clearwater, Florida, on the balmy Pinellas Suncoast. Having received a scholarship to attend Tulane University, he did so for one year. The author then transferred to the University of Florida to enter the pharmacy program. Following his graduation in early 1978, he practiced hospital pharmacy for a little more than one year, eventually becoming the assistant director of inpatient operations.

Alas, in August of 1979, after retiring from the pharmacy profession, he entered the Ph.D. program at the University of Florida, in the Department of Pharmacology and Therapeutics. With the perspicacious guidance of Professor Kenneth Leibman, Marshall's research efforts into the effects of aging on drug metabolism were both instructive and productive, and he completed the degree requirements in 1983.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kenneth C. Leibman

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Professor of Pharmacology
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Assistant Professor of
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Allen H Neims

Allen H. Neims
Professor and Chairman of
Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kathleen T Shiverick, Ph.D.
Kathleen T. Shiverick
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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